UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

ENHANCING BIOACTIVE FATTY ACIDS OF THE MEAT FROM LAMBS REARED IN INTENSIVE SYSTEMS THROUGH NUTRITIONAL MODULATION

ALEXANDRA EDUARDA AMADOR DE OLIVEIRA FRANCISCO

Orientadores: Doutor Rui José Branquinho de Bessa

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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Produção Animal

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1. É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE/TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

Faculdade de Medicina Veterinária da ULisboa, 09/05/2016

Assinatura:

Dedico esta Tese

Ao meu filho, Afonso

Ao meu marido, José Augusto

Ao *meu* avô, José Francisco

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ABSTRACT

Enhancing bioactive fatty acids of the meat from lambs reared in intensive systems through nutritional modulation

Ruminant meats are characterized by being rich in saturated fatty acids (SFA), particularly, and low in polyunsaturated fatty acids (PUFA), which is regarded as disadvantageous for humans. However, ruminant meats are also the dietary source of some bioactive health benefit fatty acids, including conjugated linoleic acid isomers (CLA) and long chain polyunsaturated fatty acids from the n-3 series (n-3 LC-PUFA). In the present thesis we have explored three nutritional strategies to improve the fatty acid (FA) profile of lamb meat, in order to maximize CLA and n-3 LC-PUFA content. Two experiments were conducted to evaluate the effects on lipid metabolism and fatty composition of meat from lambs intensively fattened of the i) dietary inclusion of *Cistus ladanifer* L. (*C. ladanifer*); ii) dietary supplementation with vegetable oils and iii) replacement of cereal grains by dehydrated citrus pulp (DCP) in diets. A global evaluation of the effects of the dietary treatments on production, meat quality and meat lipid composition, was achieved by recording the productive performance of lambs, carcasses composition, and determining analytically the fatty acid composition, oxidative stability and organoleptic quality of meat. Moreover, the expression of genes encoding Δ5-, Δ6- and Δ9-desaturases (*FADS1*, *FADS2* and *SCD*) in *longissimus* muscle was also assessed. The first experiment, involved 54 Merino Branco lambs, and evaluated the effects of the dietary inclusion of *C. ladanifer* (rockrose) (Cistus) and of the lipid supplementation with a blend of linseed and soybean oil (2:1 vol/vol). Nine diets were formulated resulting from the combination between 3 levels of *C. ladanifer* inclusion (50, 100 and 200g/kg of dry matter (DM)) and 3 levels of oil supplementation ((0, 40 e 80g/kg DM)). On second experiment, 32 Merino Branco lambs were fed using diets containing 60g/kg DM of soybean oil and 50% of dehydrated lucerne, and there were evaluated the effects of *C. ladanifer* inclusion (0 vs 150g/kg) DM) and the replacement of cereal grains by DCP in the diets. On the first experiment, the diets did not affect animal´s growth. Lipid suplementation reduced DM intake, muscle proportion in the carcass and increased kidney knobb channel fat. Meat chemical and physical parameters were not influenced by the diets. The level of 100g/kg of *C. ladanifer* inclusion improved meat oxidative stability during 7 days of storage. The perception of off-flavours was higher and meat overall acceptability by the sensory panel was reduced with suplementation 8% oil blend. The increasing inclusion of *C. ladanifer* and oil in the diet act sinergetically on the 10*t*-18:1 in meat. *C. ladanifer* increased total amount and proportion of *trans* monoinsaturated FA (*trans* MUFA) and reduced the proportions of eicosapentaenoic (EPA) and docosapentaenoic (DPA) acids. The dietary suplementation

with vegetable oil blend was a good strategy to improve nutritional value of meat, enhancing total amount of linolenic acid (18:3 n-3) and of n-3 PUFA, and reducing the proportion of SFA, which it was followed by an increment of the proportion of PUFA on lipid profile of intramuscular fat (IMF). The expression of the *SCD* mRNA increased with *C. ladanifer* but it was not correlated with the concentration of the ∆9-desaturase FA products. On the second experiment, DM intake increased with *C. ladanifer,* althought growth was not influenced by diets. Carcass composition was not affected by the dietary treatments, but an interaction between *C. ladanifer* and DCP was observed for dressing percentage. Dietary factors did not influence muscular pH, cooking losses, meat color and oxidative stability during storage. However, meat shear force increased with the adition of *C. ladanifer* to diets. Meat tenderness and juiciness were both reduced by *C. ladanifer* and DCP, but only *C. ladanifer* reduced meat overall acceptability assessed by the sensory panel. The inclusion of *C. ladanifer* to 1:1 forage to concentrate ratio diets enriched with soybean oil, reduced the occurrence of *trans*-10 shift, but did not increase 11*t*-18:1 and 9*c*,11*t*-18:2 concentrations on meat lipids. *Trans* fatty acids (TFA) were reduced by *C. ladanifer* inclusion in diets. Dehydrated citrus pulp improved meat nutritional value by increasing 18:3 n-3.

Keywords: Ruminants; meat bioactive fatty acids; *Cistus ladanifer,* lipid supplementation; dehydrated citrus pulp

RESUMO

Enriquecimento em ácidos gordos bioactivos da carne de borrego de sistema intensivo através da modulação nutricional do metabolismo lipídico

A carne de ruminantes caracteriza-se por ser particularmente rica em ácidos gordos saturados (AGS) e pobre em ácidos gordos poliinsaturados (AGPI), o que é considerado como negativo para a dieta humana. Contudo, é também uma importante fonte de alguns ácidos gordos bioactivos benéficos para a saúde, como os isómeros conjugados do ácido linoleico (CLA) e ácidos gordos poliinsaturados de cadeia longa da família n-3 (n-3 CL-AGPI). Na presente tese explorámos três estratégias nutricionais de modo a melhorar o perfil lipídico da carne de borrego através do aumento do seu conteúdo em CLA e em n-3 n-3 CL-AGPI. Foram realizadas duas experiências nas quais os efeitos i) da inclusão de *Cistus ladanifer* L. (esteva); ii) da suplementação lipídica com óleos vegetais e iii) da substituição dos grãos de cereais por polpa de citrínos desidratada (PCD) no metabolismo lipídico e na composição em ácidos gordos da carne de borrego de sistema intensivo de engorda foram avaliados. A avaliação integrada dos efeitos dos tratamentos no desempenho produtivo, foi efectuada registando-se a ingestão de matéria seca (MS), ganho médio diário e composição das carcaças dos borregos, assim como a qualidade física e sensorial da carne e a sua composição em ácidos gordos e níveis de expressão dos genes que codificam para as Δ5, Δ6 *e* Δ9-desaturases (*FADS1*, *FADS2* e *SCD* mRNAs). No primeiro ensaio, que envolveu 54 borregos Merino Branco avaliámos a inclusão, na dieta, de *Cistus ladanifer* (esteva), e da suplementação lipídica constituída por uma mistura de óleo de linho e de soja (2:1 vol/vol). Para tal foram formuladas 9 dietas que resultaram da combinação entre 3 níveis de inclusão de *C. ladanifer* (50, 100 e 200g/kg de matéria seca (MS)) e 3 níveis de suplementação lipídica (0, 40 e 80g/kg de MS). No segundo ensaio, utilizaram-se 32 borregos alimentados com dietas contendo 50% de luzerna desidratada e 60g/kg MS de óleo de soja, e avaliaram-se os efeitos da inclusão de *C. ladanifer* (0 *vs* 150g/kg) de MS) e da substituição dos grãos de cereais por polpa de citrínos desidratada (PCD) nas dietas. No primeiro ensaio, os tratamentos não afectaram o crescimento dos animais. O óleo reduziu a ingestão de MS, a proporção de músculo da carcaça e aumentou a quantidade de gordura pélvica e renal. As caraterísticas físicas e químicas da carne não foram afectadas pelas dietas. O nível de 100g/kg de esteva melhorou a estabilidade oxidativa da carne durante o armazenamento de 7 dias. A percepção de off-flavours foi superior e a aceitação global por parte do painel de provadores foi inferior para a suplementação com 8% de óleo. A inclusão de *C. ladanifer* potenciou o aumento de 10*t*-18:1 na carne induzido pelo óleo. Com a inclusão de *C. ladanifer* nas dietas aumentou a quantidade total e proporção de ácidos gordos

monoinsaturados *trans* (*Trans*AGMI) e diminuiram as proporções dos ácidos eicosapentaenoico (EPA) e docosapentaenoico (DPA). A suplementação das dietas com a mistura de óleos vegetais foi uma boa estratégia para melhorar o valor nutricional da carne, permitindo um aumento da quantidade total de ácido linolénico (18:3 n-3) e n-3 AGPI, e a redução da proporção de AGS acompanhada de um aumento da proporção de AGPI no perfil lipídico da gordura intramuscular. A expressão do mRNA do gene *SCD* aumentou com a inclusão de *C. ladanifer* nas dietas. No segundo ensaio, a ingestão de MS aumentou com a inclusão de *C. ladanifer,* embora o crescimento não tenha sido influenciado pelas dietas. A composição da carcaça não foi afectada pelos tratamentos, mas verificou-se uma interacção entre a inclusão de esteva e a de PCD que afectou o rendimento da carcaça. Os factores em estudo não influenciaram o pH muscular, as perdas por cozedura, a cor da carne e a sua estabilidade oxidativa. No entanto, ocorreu um aumento da força de corte da carne com a adição de *C. ladanifer* às dietas. A tenrura e a suculência da carne sofreram uma diminuição com a inclusão de *C. ladanifer* e de PCD nas dietas, no entanto a aceitação global da carne pelo painel de provadores apenas foi diminuída com *C. ladanifer.* A inclusão de *C. ladanifer* a dietas com uma relação de forragem: concentrado de 1:1 e enriquecidas com óleo de soja, reduziu a ocorrência do *trans*-10 shift, embora não tenha aumentado os teores de 11*t*-18:1 e de 9*c*,11*t*-18:2 na fracção lipídica da carne. O total de ácidos gordos *trans* (AGT) na carne foi reduzido pela inclusão de esteva nas dietas. A PCD melhorou o valor nutricional da gordura da carne de borrego, aumentando a concentração em 18:3 n-3.

Palavras chave: Ruminantes; ácidos gordos bioactivos da carne; *Cistus ladanifer*; suplementação lipídica; polpa de citrinos desidratada

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a*	Redness (colour dimension)
AA	Araquidonic acid, 20:4 n-6
ACACA	Acetyl-CoA carboxylase
ADG	Average daily gain
Ag+-HPLC	Silver high performance liquid chromatography
ALA	Linolenic acid, 18:3 n-3, 9*c*,12*c*,15*c*-18:3
AOAC	Association of Official Analytical Chemists
b*	Yellowness (colour dimension)
BCFA	Branched-chain fatty acids
BD	Bolsa de doutoramento
BHT	Butylated Hydroxytoluene
BI	Biohidrogenation intermediates
c	*cis*
C*	Colour saturation
CCW	Cold carcass weight
CHD	Coronary heart disease
CIE	Comission Internationale de l'Eclairage
CIISA	Centro de Investigação Interdisciplinar em Sanidade Animal
Cistus	*Cistus ladanifer*
CP	Citrus pulp
CLA	Conjugated isomers of linoleic acid
CL	Cooking losses
CoA	Coenzyme A
CT	Condensed tannins
CV	Coefficient of variation
CVD	Cardiovascular disease
CW	Carcass weight
d	Day
DAD	Diode array detector
DCP	Dehydrated citrus pulp
DeoxyMb	Deoxygenated myoglobin
DHA	Docosahexaenoic acid (22:6*n-3*)
DM	Dry Matter
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid (22:5*n-3*)
EC	Enzyme Commission number
EDTA	Ethylenediaminetetraacetic acid disodium salt
EFSA	European food safety authority
ELOVL2	Fatty acid elongase 2
ELOVL5	Fatty acid elongase 5
e.g.	*exempli gratia*
EPA	Eicosapentaenoic acid (20:5*n-3*)
ES	Energy source
et al.	and others
FA	Fatty acids

FADS1	Fatty acid desaturase 1
FADS2	Fatty acid desaturase 2
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization of the United Nations
FAO/WHO	Food and Agriculture Organization/World Health Organization
FASN	Fatty acid synthase
FCT	Fundação para a Ciência e a Tecnologia
FID	Flame ionization detector
FMV	Faculdade de Medicina Veterinária
GC	Gas chromatography
GPP	Gabinete de planeamento e política
GRAS	Generally recognized as safe
H*	Hue angle
h^2	Heritability
HCl	Hydrochloric Acid
HCW	Hot carcass weight
HDL	High density lipoprotein
HPLC	High-performance liquid chromatography
HNE	4-hydroxynonenal
IL-6	Interleukine-6
IMF	Intramuscular fat
INE	Instituto Nacional de Estatística
INIAV	Instituto Nacional de Investigação Agrária e Veterinária
iTFA	*Trans* fatty acids from industrial origin
KKCF	Kidney knobb channel fat
L*	Lightness (colour dimension)
LA	Linoleic acid, 18:2*n*-6, 9*c*,12*c*-18:2
LC	Liquid chromatography
LC-PUFA	Long-chain polyunsaturated fatty acid
LDL	Low density lipoprotein
LSMEANS	Least squares means
LL	*Longissimus lumborum*
LPL	Lipoprotein lipase
LT	*Longissimus thoracis*
MB	Merino Branco
MDA	Malonaldehyde
MetMb	Metamyoglobin
mRNA	Messenger ribonucleic acid
MUFA	Monounsaturated fatty acid
NDF	Neutral detergent fiber
NEFA	Non esterified fatty acid
NL	Neutral lipids
ns	Not significant
n-3	Omega 3
n-6	Omega 6
n-6/n-3	Total omega 3 fatty acids/total omega 6 fatty acids ratio
O	Oil
P	Probability
PCR	Polymerase chain reaction
pH	Potential of hydrogen

PL	Polar lipids
PUFA	Polyunsaturated Fatty Acids
r	Correlation coefficient
r^2	Determination coefficient
RBH	Ruminal biohydrogenation of dietary polyunsaturated fatty
RDI	Recommended daily intake
RNA	Ribonucleic acid
rTFA	*Trans* fatty acid from ruminal origin
RT-qPCR	Reverse transcriptase – polymerase chain reaction
SAS	Statistical analysis system
SCD	Stearoyl-CoA desaturase
SD	Standard deviation
SEM	Standard error of the mean
SF	Shear force
SFA	Saturated fatty acids
SREBF1	Sterol regulatory element binding factor 1
SREBP	Sterol regulatory element binding protein
SW	Slaughter weight
T	Temperature
t	*Trans*
TAG	Triacylglycerols
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TFA	*Trans* fatty acids
UFA	Unsaturated fatty acids
UK	United Kingdom
UEISPA	Unidade Estratégica de Investigação e Serviços em Produção
USA	United States of America
USDA	United States Department of Agriculture
UV	Ultraviolet
VFA	Volatile fatty acids
vs.	*Versus*
VLDL	Very low density lipoproteins
VO	Vegetable oil

LIST OF SYMBOLS AND UNITS

%	Percent
±	Standard deviation/Standard error/Standard error of the mean
<	Less than
>	Greater than
ºC	Degree Celsius
cm	Centimetre
cm^2	Square centimeters
cm^3	Cubic centimeters
g	Gram
h	Hour
kcal	Kilo calories
kg	Kilogram
M	Molar
mg	Miligram
mL	Mililitre
min	Minute
mm	Milimetre
n	Number of experimental units
rpm	Revolutions per minute
s	Second
vol/vol	Volume per volume

INTRODUCTION

Ruminant meats are characterized for being rich in SFA and low in PUFA, which is regarded as negative for the human health. However, they are simultaneously the major dietary source of the conjugated isomers of linoleic acid (18:2 n-6) (CLA) and an important source of long chain (C≥20) polyunsaturated fatty acids (PUFA) from the n-3 family (n-3 LC-PUFA), such as eicosapentanoic (EPA, 20:5 n-3), docosapentaenoic acid (DPA, 22:5 n-3) and docosahexaenoic (DHA, 22:6 n-3) acids. This fact assumes an especial nutritional relevance in human populations with a high intake of meat from ruminants.

The CLA acronym refers to the group of positional and geometric isomers of linoleic acid, where the double bonds are conjugated. Conjugated linoleic acid has been recognized as exerting potent anticancerinogenic effects (Wahle, Heys & Rotondo, 2004). Ruminant fats, due to the ruminal biohydrogenation (RBH) of dietary PUFA, are the main natural sources of CLA in human diet, particularly of rumenic acid (9*c*,11*t*-18:2), which is the major CLA isomer present in meat and milk from ruminants, comprising in general, more than 80% of the total of CLA isomers (Khanal & Dhiman, 2004). However, most of the 9*c*,11*t*-18:2 deposited in tissues does not result directly from RBH, but it is derived from the endogenous conversion of vaccenic acid (11*t*-18:1) by the enzyme stearoyl CoA desaturase (SCD or Δ9-desaturase) (Palmquist, St. Pierre & McClure,2004). Therefore, the potential increase of 9*c*,11*t*-18:2 in meat of ruminants necessarily involves an high rumen 11*t*-18:1 outflow and a high Δ9-desaturase activity (Bessa, Alves & Santos-Silva, 2015). Feeding growing ruminants with diets rich in forage allows the increase of 11*t*-18:1 availability to its endogenous conversion in 9*c*,11*t*-18:2 (Bessa *et al*., 2015). However, in this type of diets, the expression of the Δ9-desaturase gene (*SCD*) is down-regulated due to a low starch content and low insulin levels, what may limit the synthesis of 9*c*,11*t*-18:2 in tissues (Daniel, Wynn, Salter & Buttery, 2004; Bessa *et al.*,2015). Moreover, since 9*c*,11*t*-18:2 is mostly deposited in triglycerides (TAG) (Jerónimo *et al*., 2011), a high intramuscular fat (IMF) deposition is also required (Bessa *et al.*, 2015). This fact represents an important constrain to CLA deposition in the meat of ruminants fed with high levels of forage, due to the low energy content of these diets that limits the intramuscular fat deposition (Bessa *et al*., 2015).

Cereal-rich concentrate feeds are currently used in the diet of growing ruminants and as concentrates have high energy content, mainly due to the starch from the cereal grains, they promote high insulin levels and high TAG deposition. However, the occurrence of changes in rumen environment modifies the normal pattern of RBH, favoring the synthesis and accumulation on rumen of 10*t*-18:1 instead of 11*t*-18:1 (*trans*-10 shift). As 10*t*-18:1 cannot be converted in 9*c*,11*t*-18:2 its deposition in IMF is compromised. In such conditions, the *trans*-

10 shift is considered as the main constrain to enhancing the concentration of CLA in the meat of concentrate-fed ruminants (Bessa *et al.*, 2015).

The beneficial health effects of the n-3 LC-PUFA are well documented and include anti-atherogenic, anti-thrombotic and anti-inflamatory effects (Calder, 2012). Increase the intake of EPA, DPA and DHA in the diet is an important concern in human nutrition since the endogenous synthesis of these FA from linolenic acid (18:3n-3) is very limited (Burdge & Calder, 2005). The main sources of EPA and DHA for the human diet are the marine products, particularly the oil-rich fish (Calder, 2012). However, in populations where the intake of sea food is very low but that of meat from ruminants is high, this meat can be a valuable source to the dietary intake of n-3 LC-PUFA, (Givens, Kliem & Gibbs, 2006; Givens, 2009). Forages are one of the most important sources of 18:3 n-3 in ruminant diets (Raes, De Smet & Demeyer, 2004) and despite of RBH effects, it is well established that feeding ruminants with forage-rich diets, especially with fresh grass, results in a higher content of 18:3 n-3 and its longer chain derivatives, mainly EPA, in meat (Fisher *et al.*, 2000; Santos-Silva, Bessa & Santos-Silva, 2002a; Bessa, Portugal, Mendes & Santos-Silva, 2005; Bessa, Lourenço, Portugal & Santos-Silva, 2008; Alfaia *et al.*, 2009). Diets rich in starch tend to reduce the proportion of dietary PUFA which is biohydrogenated by the rumen microorganisms, allowing that a high proportion of those FA is available to absorption and deposition in tissues (Glasser, Schmidely, Sauvant & Doreau, 2008). However, and mainly due to its high content in grains, concentrate feeds are rich in 18:2 n-6 and as consequence, the meat from ruminants fed with concentrate-based diets presents a high level of n-6 FA, including 18:2 n-6 and its longer chain derivatives (n-6 LC-PUFA) (Wood *et al.*, 2008). Nevertheless, the supplementation of high-grain diets with n-3 PUFA sources allows that a higher proportion of those FA escape from RBH and be deposited in IMF, thus, increasing 18:3 n-3 and n-3 LC-PUFA content in the meat from feedlot ruminants (Sinclair, 2007; Scollan *et al.*, 2014; Alvarenga, Chen, Furusho-Garcia, Perez & Hopkins, 2015).

In the present thesis we intented to explore three nutritional strategies in order to enhance the content on 11*t*-18:1 and CLA, namely the isomer 9*c*,11*t*-18:2 and on *n*-3 LC-PUFA of meat from lambs fed with forage-concentrate basal diets (50:50). Those strategies were evaluated in two experiments and were: a) the dietary inclusion of a Mediterranean shrub (*Cistus ladanifer* L.) (Experiments 1 and 2); b) the lipid supplementation with vegetable oils as PUFA sources (Experiments 1 and 2) and c) the replacement of the cereal grains of the concentrate fraction of the diets by dehydrated citrus pulp (DCP) as an alternative energy source (Experiment 2). Considering an integrative approach, we analised the effects of each strategy on the productive performance of lambs, on carcass characteristics, on meat quality, including sensorial analysis and meat colour and lipid oxidative stability during 7 days of storage. Furthermore, we also evaluated the effects of each strategy on the expression of the

genes that codify for Δ9, Δ5 and Δ6-desaturases (*SCD*, *FADS1* and *FADS2*, respectively), which are directly related to the endogenous synthesis of 9*c*,11*t*-18:2 and LC-PUFA.

This document is organized in 7 Chapters where, after the Bibliographic Review (Chapter 1), we will present the results of each of the two experiments performed, in the form of scientific publications (Chapters 2 to 5). In the Chapter 6 (General discussion) we will discuss the results obtained for each of the three dietary strategies which were explored and evaluated during this work. Finally, in the Chapter 7 (Conclusions, implications and future perspectives) the main general conclusions of this thesis and some final considerations will be provided.

CHAPTER 1

Scientific background and objectives

1. 1. Production of sheep meat

1.1.1. Sheep demographical data

Sheep is a species with a high adaptation capacity to climatic, topography and dietary adverse conditions. Besides, it is a “multi-product” species providing income from wool, meat and milk (Sañudo, Sanchez & Alfonso, 1998a).The number of sheep in the world has remained around one billion in the last five decades and in 2013 the world ovine population was of 1 162 875 535 animals (FAOSTAT,http://faostat3.fao.org/,2015). China, Australia and India had, respectively, 15%, 6.5% and 5.5% share of the world population. With 97 773.5 million animals, the European Union (EU-28) held 8.4% share of the world sheep flock (FAOSTAT,http://faostat3.fao.org/, 2015).

As consequence of the mid-term review of the Common Agricultural Policy of EU (CAP) which leaded to the decoupling of the subsidies in 2003, the total numbers of livestock in EU-28 decreased for the various livestock species. Between 2005 and 2013 bovine and pigs population decreased 3.0% and 8.6%, respectively, for the EU-28. Numbers of goats fell 2%, for the five Member States with more than 500 000 goats and sheep fell 11.6% for the 14 Member States with more than 500 000 sheep, corresponding to a loss of 10.96 million sheep (Figure 1.1)(Eurostat, 2014b).

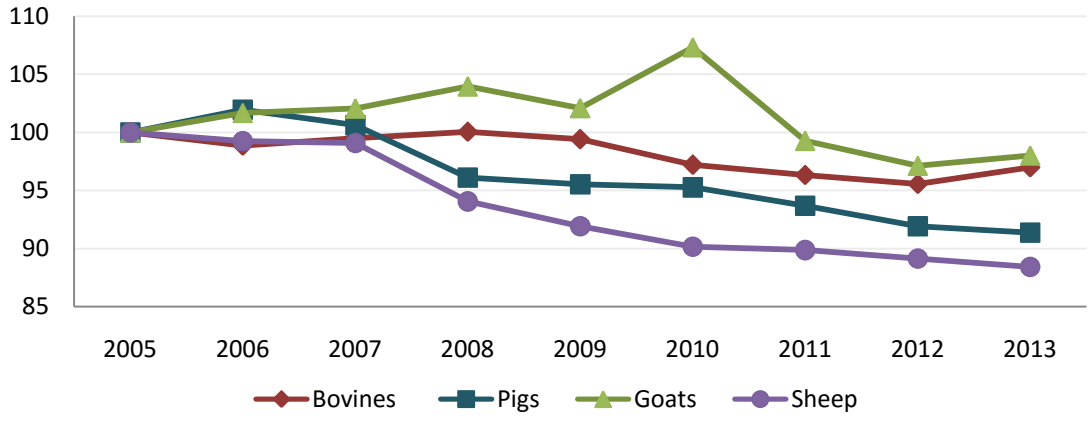


Figure 1.1 -Changes on EU-28 livestock populations between 2005 and 2013 (Index 2005=100). Adapted from Eurostat (2014b). Data of sheep and goat populations are from the EU-28 members with at least 500 000 heads

Portugal was the country with the major reduction on its sheep flock between 2005 and 2013. There was a reduction of 829 350 heads, corresponding to a decrease of 29% on the Portuguese flock in those 9 years (Figure 1.2). Also Spain, Ireland and France have lost

important proportions of their sheep effective between 2005 and 2013 (28, 24 and 18%, respectively). The United Kingdom (UK) which is the most important producer of sheep meat in EU-28, have lost 4.7% of its flock (Eurostat, 2014b).

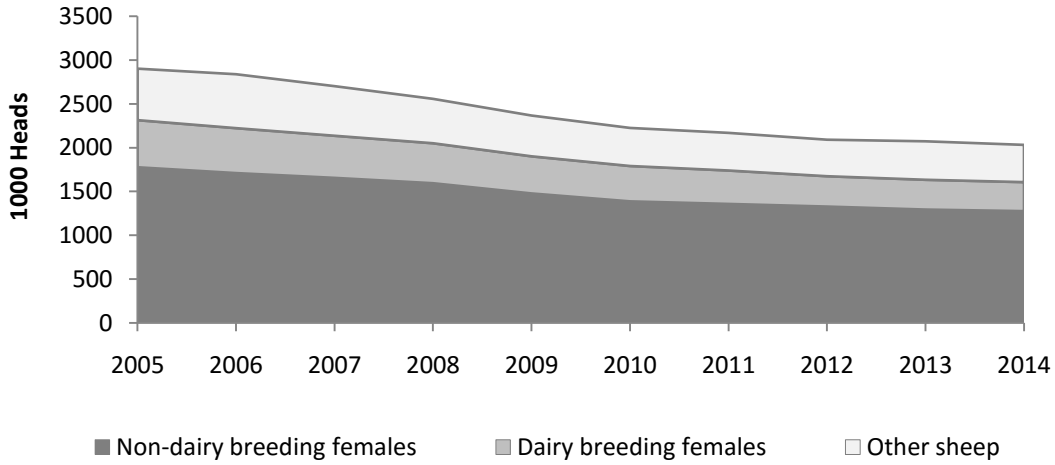


Figure 1.2 – Evolution and composition of Portuguese sheep flock between 2005 and 2014. Data source: EUROSTAT,http://ec.europa.eu/ (2015).

In 2013, the countries of Europe (EU-28) with higher sheep flocks were UK, with 22,624 million heads (26% of the EU-28 sheep flock) and Spain, with 16,119 million heads (19% of the EU-28 sheep flock). Portugal held 2,074 million animals (3% of the EU-28 sheep flock) (Figure 1.3) (Eurostat, 2014b).

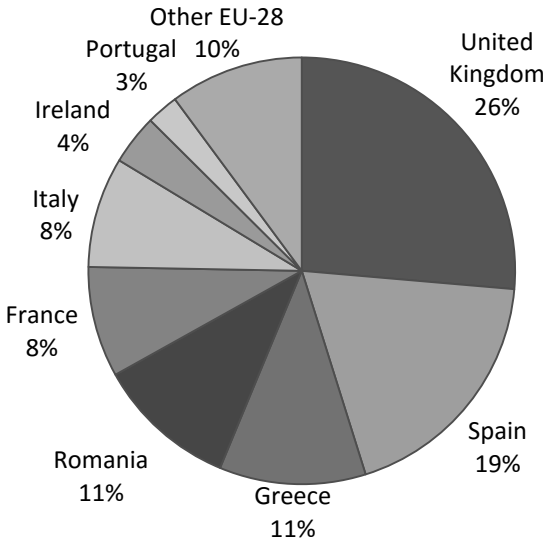


Figure 1.3 - Distribution of sheep in EU-28 in 2013. Adapted from Eurostat (2014b).

Considering the most recent statistical data, in 2014, the Portuguese sheep flock was composed by 2 032 620 animals, what represents 33.4% of the total of national livestock population (cattle, sheep, goats and pigs). From those, 62.8% were non-dairy breeding females, 16.3% were dairy breeding females and 20.9% of the animals where rams, lambs for meat production and ewe-lambs for flock renewal (other sheep category) (Figure 1.2). Alentejo is the region that holds the largest sheep population, (56.6% of the national flock), with 1 150 780 heads (EUROSTAT,http://ec.europa.eu/, 2015; INE, 2015).

1.1.2. Sheep meat production and consumption

In 2013, a total of 310,379,920.30 tons of meat were produced in the world.From those, 2.8% were from ovine (8,589,257.17 tons). China was the major sheep meat producer with 2,081,000.23 tons, followed by EU-28 that produced 853,514.60 tons. Australia comes in third with a production of 660,437.00 tons of sheep meat and New Zealand in fourth (450,075.00 tons). New Zealand, Australia and UK are the world major exporters of sheep meat (348,716.00 tons, 311,696.00 tons and 94,660.00 tons, respectively), while the major importers are the European Union (EU-27) (153,211.00 tons) and China (152,785.00 tons) (data available for 2012) (FAOSTAT,http://faostat3.fao.org/, 2015).

The production of sheep meat represented in 2013, 1.9% of the total of EU-28 meat production that it was of 44,355,494.50 tons (FAOSTAT,http://faostat3.fao.org/, 2015). Contrarily to the reduction of 493,272.40 tons (-36.6%) observed on sheep meat production in EU between 1991 and 2012, (FAOSTAT,http://faostat3.fao.org/, 2015), between 2012 and 2013 the EU-28 production of sheep meat was increased in 0.8% (Eurostat, 2014a). The UK and Spain that are the most important sheep meat producers of EU-28, produced, in 2013, respectively, 35.4% and 14.4% of the EU-28 sheep meat (Eurostat, 2014a). However, it should be taken in account that 109,700.00 tons of sheep meat (13% of total sheep meat produced in EU-28) comes from domestic slaughtering. This is a marginal phenomenon that primarily arises from own-consumption, and traditional or ritual slaughter and it has a higher expression in those Member States that joined to EU in 2004, influencing the statistical data associated to the slaughtering and meat production in EU-28. Although the tendency to decrease this type of slaughtering in the last years, due to the hygiene and food safety rules of EU, it depends on the country and of the livestock species. In the case of Portugal, in 2013, 39.3 % of all the sheep meat produced was associated to the domestic slaughtering (Eurostat, 2014b). Considering the meat from livestock animals slaughtered in the slaughterhouses and approved to consumption, during the year of 2014 it was produced in Portugal a total of 451,369 tons of bovine, goat, sheep, pig and equidae meat, which of 10,222 tons (2.26%) was from sheep meat. The year of 2014 reflected the trend observed in

2013 for a slight recovery on sheep slaughtering in Portugal (+ 3.86% of heads comparing to 2012). It were slaughtered 887,619 ovine animals and the average carcass weight was of 11.5kg. Lambs producing carcasses with 10kg or more were the most frequent (58% of total slaughters), contributing to 66.1% of total sheep meat production. Milk lambs (producing carcasses with less than 10kg) contributed to 20.3% of the sheep meat produced and adults to 13.6%. The Centro (41.0%) and Alentejo (32.8%) were the Regions where occurred most of the sheep slaughtering (INE, 2015).

Due to cultural, economic and gastronomic practices, in Portugal the sheep and goat meats consumption is mostly concentrate in Christmas and Easter, with a small peak in summer months. In 2014, the *per capita* consumption of both sheep and goat meats was of 2.3 kg/inhab/year, what represents a reduction of 4.2% relatively to 2012 and 2013, confirming the decline observed since 2008. Portugal has a high degree of self-sufficiency in sheep and goat meats, which is around 80% (79.2% in 2014, 80% in 2013 and 76% in 2012) (INE, 2015). Spain (2,579 tons), UK (1,206 tons) and New Zealand (1,026 tons) are the major supplier countries of sheep meat for the Portuguese market (data from 2014,www.trademap.org, 2015).

1.1.3. Production systems of sheep meat in Portugal

Sheep meat is traditionally regard by consumers as a natural product, produced by lambs reared by their dams in grasslands, within an extensive production system of low impact on the environment and associated to the concept of animal welfare (Sañudo *et al*., 1998a) .However, there is a high diversity of exploration systems associated to sheep farming that results from the interaction between several factors such as the sheep breed aptitude (dairy or meat), the exploration productive objectives, the availability of land, labor force, feed resources (pastures, forage resources and concentrate feeds) and the capacity for system intensification. This variability associated to production systems peculiarities leads to the several types of final product from animals with variable age, slaughter weight, feeding system and breed aptitude that can be observed, not only globally, but at a regional level as within EU-28. The countries from the North of Europe are typically sheep meat producers while the countries of the South present a significant dairy production (Table 1.1) (Eurostat, 2014b). This difference in the farms aptitude is reflected in the carcass weight of lambs traditionally produced and preferentially consumed in each country and region of Europe. Thus, in the Central-Northern countries, meat from heavy animals is preferred by the consumers and in contrast, in the Mediterranean-Southern countries, where the dairy production is more expressive, the consumer´s preference goes for lambs with very young age (1-4 months) and lower carcasses weights (Sañudo *et al.*, 1998a). Also the feeding

system of lambs is other important factor that influences the consumer´s preferences of meat flavour and overall acceptability as it was observed by Sañudo *et al.* (1998b) in a study where sensorial panels from Spain and United Kingdom assessed meat from lambs produced in both countries.

Table 1.1 – The national ewe flocks of the14 countries of EU-28 with more than 500 000 sheep and their dairy share in 2014.

	Number of Ewes (1000 heads)			Dairy ewes (%)
	Total Ewes	Dairy	Non-dairy	
Northern countries	**19 900.2**	**38.7**	**19 860.5**	**0.2**
United Kingdom	14 849.0	0.0	14 849.0	0
Ireland	2 472.8	0.0	2 472.8	0
Germany	1 116.4	10.7	1 105.7	1.0
Hungary	889.0	22.0	866.0	2.5
Netherlands	573.0	6.0	567.0	1.0
Sweden	-	-	-	0
Southern countries	**40 808.6**	**24 622.2**	**17 186.5**	**58.9**
Spain	11 947.7	2 583.3	9 364.4	21.6
Romania	7 875.4	7 078.0	797.4	89.9
Greece	6 733.0	6 449.0	284.0	95.8
Italy	6 322.9	5 246.5	1 076.4	83.0
France	5 535.0	1 595.0	3 940.0	28.8
Bulgaria	1 259.5	1 170.2	89.3	92.9
Portugal	1 634.2	340.2	1 294.0	20.8
Croatia	501.0	160.0	341.0	31.9

Adapted from Eurostat (2014b).

In Portugal, the principal sheep farming systems (dairy, meat extensive and intensive systems) produces four main types of sheep meat. The **milk or suckling lambs** are young lambs that come from the dairy production system. These lambs are traditionally reared in exclusive with their dam's milk until they reach 1-1.5 months of age. At that time, they are slaughtered, producing carcasses with 4-9 kg weight. Due to the intensification of the sheep dairy system, artificial rearing of lambs and supplementation with milk-concentrates are more frequent. These lambs come from Portuguese dairy breeds, exotic dairy breeds and their crosses. The "Borrego da Serra da Estrela" is the only milk lamb meat with Protected Geographical Indication which is actually in the market (GPP, 2014).The **pastoral or grazing lambs** come from the extensive meat production system based on grazing. Traditionally, these lambs are reared with their dams on pasture, complementing the dam's milk with grazing, until they reach the 4-5 months of age and 20-25kg of live weight. By that time, they are slaughtered producing carcasses weighing 10-15kg. Most of the lamb meat produced in

Portugal comes from this system, being the Merino Branco breed and its crossbreedings with other meat-type breeds the most used type of lamb. The “Borrego do Nordeste Alentejano” is reared in Alentejo Region and it is the only lamb meat with Protected Geographical Indication (GPP, 2014) actually in the market that is produced by this system. Actually, and in order to obtain carcasses with higher level of fatness and meat with good organoleptic quality, the supplementation of the grazing lambs with conventional concentrates during a finishing period is a common practice among farmers. However, the supplementation of lambs with concentrated feed may present a negative impact on meat nutritional value, due to the effects on the fatty acid profile of intramuscular fat (IMF), namely the reduction of the proportions of fatty acids (FA) with beneficial health effects such as the polyunsaturated fatty acids of the *n*-3 series (*n*-3 PUFA) and CLA (Santos-Silva *et al.*, 2002a). The **feedlot lambs or intensive system fattening lambs** result from the intensification of the sheep meat production system. Lambs are reared with their dams until weaning with about 42-60 days of age. After weaning, are fed in confinement with commercial concentrate during 6-7 weeks, being slaughtered with 3-4 months of age and 25-35kg of live weight. The carcasses produced weigh around 13-15kg. In Portugal, this product only represents a low proportion of the sheep meat produced and is mostly for the supply of the larger retailers (super and hypermarkets). For the retail, this system reduces the variability found in the final product associated to differences in the lambs feeding system, thus producing more homogeneous carcasses, with a higher degree of fatness and quality. Finally, the **adult sheep** meat comesfrom both milk and meat production systems and is mostly meat of adult animals in the end of productive cycle.

1.2. Effects of dietary fat on Human health, lipid composition of ruminant´s meat and international guidelines for fat intake

In the last years the relationship between diet and the development of chronic disease is being more evident, due to several clinical and epidemiological studies (World Health Organization [WHO], 2003; Food and Agriculture Organization of the United Nations [FAO], 2010). Cardiovascular disease (CVD), cancer, diabetes type II, due to insulin resistance and obesity are some of the chronic diseases that are currently associated to fat intake (level and quality) (WHO, 2003; Givens *et al.*, 2009; FAO, 2010). The high prevalence of these diseases in the western countries is responsible for substantial direct and indirect economic costs and for millions of deaths each year. Data from the technical report of WHO (2003) on “Diet, nutrition and the prevention of chronic diseases” suggested that by 2020, almost 75% of all deaths worldwide will be caused by chronic diseases, especially by CVD. Thus, global public health policies were developed, with emphasis on the reduction of total fat, saturated fatty acids (SFA) and *trans* fatty acids (TFA) intake, being recommended the replacement of

dietary SFA and TFA by polyunsaturated fatty acids (PUFA) namely n-3 LC-PUFA. That process resulted in the establishment by several public organisms around the world, of dietary guidelines for the intake of total fat and of the main fatty acids classes (Table 1.2).

Table 1.2 – Recommended values for the dietary intake of total fat and of fatty acids for healthy adults.

Total fat or fatty acid intake	Organism / country			
	FAO[1]	U.S.A.[2]	Britain[3,4]	Australia and New Zealand[5]
Total Fat	20-35% E[a]	20-35% E	33% E	20-35% E
SFA	<10% E	<10% E	10% E	-
Trans MUFA	<1% E	-	2% E	-
PUFA	6 – 11% E	6 -11% E	6.5 % E	-
n-3 PUFA	0.5-2% E	-	-	-
18:3*n*-3	>0.5% E	0.6 – 1.2% E	0.7% E	0.4 - 1% E
EPA+DHA	>250 mg/day	250mg/day	500 mg/day	0.2%* E
n-6 PUFA	2.5 - 9%E	-	-	-
18:2*n*-6	>2.5 % E	5 – 10% E	2% E	4- 10% E

[a]-Percentage of total energy intake. [*]- recommendation for EPA+DPA+DHA intake.References: [1]- Food and Agriculture Organization of United Nations (2010);[2]- United States Department of Agriculture and United States Department of Health and Human Services (2010); [3] - British Nutrition Foundation (2004); [4]- International Society for the Study of Fatty Acids and Lipids (2004); [5]- National Health and Medical Research Council (2006).

The ruminal biohydrogenation (RBH) of dietary PUFA by symbiotic rumen microorganisms leads to the high proportion of SFA, the variable amount of TFA and the low level of PUFA that characterizes the lipid profile of ruminant´s edible fat. Therefore, the consumption of products from ruminants such as milk and meat is regarded as prejudicial for human health (Givens, 2010) contributing to a negative image among consumers that have resulted, for instance, in the reduction of consumption of ruminant´s meat in several developed countries (McNeill, 2014).

Saturated fatty acids

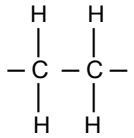
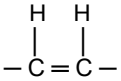
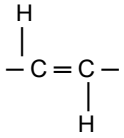
Nutritional recommendations indicate that SFA should represent a maximum of 10% of energy intake (Table 1.2), which for a normal range of daily energy intake for adults (2000 to 3500 kcal), corresponds to 24 to 36 g of SFA. Dietary SFA have been associated to the increase of CVD, particularly of coronary heart disease (CHD), due to the consistent evidence that SFA increase the concentration of serum low-density lipoprotein cholesterol (LDL-cholesterol) (Givens, 2009). Also, insulin resistance, which is directly related to metabolic syndrome and to diabetes type II, increased with SFA consumption (Funaki, 2009; Kennedy, Martinez, Chuang, LaPoint & McIntosh, 2009).

However, in relation to the effect of SFA on serum cholesterol levels and increased risk of CVD and CHD, it may seem that it depends on individual effects of each saturated fatty acid. Research has demonstrate that the dietary stearic acid (18:0), which is generally the SFA in major proportion on ruminants meat, has a neutral or even slight lowering effect on LDL-chrolesterol (Hunter, Zhang & Kris-Etherton *et al.*, 2010). In the other hand, 18:0 is converted in the tissues by the enzyme Δ9-desaturase (or stearoyl CoA desaturase- SCD) in oleic acid (9*c*-18:1) (Daniel *et al.*, 2004), which is associated to the reduction of LDL-cholesterol and the total/HDL-cholesterol ratio (FAO, 2010). In fact, in 2003, the WHO in the report on "Diet, nutrition and prevention of chronic diseases" have already considered the SFAs from 12:0 to 16:0 as cholesterol-raising fatty acids, but not 18:0. Moreover, in the last few years, the current dietary guidelines related to the consumption of SFA have been questioned by many studies, and reports that SFA intake is not clearly associated with an increased risk of cardiovascular disease are increasing (Lawrence, 2013; Lamarche & Couture, 2014; Chowdhury *et al.*, 2014; Souza *et al.*, 2015), what is causing high controversy and is under intensive debate within the scientific and medical communities.

Trans fatty acids and conjugated isomers of linoleic acid (CLA)

Trans fatty acids are unsaturated FA characterized by the presence of at least one double bond in the *trans* configuration instead the usual *cis* configuration (Table 1.3) (Dhaka, Gulia, Ahlawat & Khatkar *et al.*, 2011). *Cis* configuration of the double carbon-carbon bond causes a bend in the fatty acid chain, whereas *trans* configuration straightens it. Thus, as the *trans* bond imparts a chemical structure more similar to that of saturated FA, the physiological properties and effects of TFAs became more similar to the ones of SFA than to those of unsaturated fatty acids (UFA) (Mozaffarian, Katan, Ascherio, Stampfer & Willett *et al.*, 2006).

Table 1.3 - Types of fatty acids illustrating *trans* configuration

Types of Fatty Acids		
Saturated Fatty acid	**Unsaturated Fatty acid *cis* configuration**	**Unsaturated Fatty acid *trans* configuration**
		
Carbon-Carbon single bond	Carbon-Carbon double bond Hidrogen atoms are on the same side of the carbon-carbon double bond	Carbon-Carbon double bond Hidrogen atoms are on opposite sides of the carbon-carbon double bond

Adapted from Dhaka *et al.* (2011)

The major dietary sources of TFA are the products that integrate on their composition partially hydrogenated vegetable oil obtained by industrial processes. These include bakery products (bread, cakes, cookies), margarines, fried potatoes, potato and corn chips, popcorn, breakfast cereals and candy as examples. Ruminant products (meat, milk and by-products) are the other dietary source of TFA (Dhaka *et al.*, 2011; Tardy, Morio, Chardigny & Malpuech-Brugère, 2011). The TFA with ruminant origin (rTFA) are naturally produced by the RBH. The intake of TFA has been strongly associated to deleterious effects in human health, mainly as enhancers of the risk of CVD (Williams, 2000; Mozaffarian *et al.*, 2006; Dhaka *et al.*, 2011; Tardy *et al.*, 2011; Salter, 2013). Moreover, increased risk of insulin resistance and predispose for type II diabetes, obesity, cancer are also associated to TFA intake (Dhaka *et al.*, 2011; Tardy *et al.*, 2011). However, it seems that the biological effects of this class of FA is isomer-specific (Wang, Jacome-Sosa & Proctor, 2012; Salter, 2013) and the TFA isomers that potentially have the most negative impact in human health are the *trans*-9 18:1 (elaidic acid), which is generated in high amount during the industrial hydrogenation (Tardy *et al.*, 2011) and the *trans*-10 18:1 which is the main *trans*-octadecenoic isomer produced in the rumen of animals fed with grain-rich diets (Bessa *et al.*, 2005; Aldai, Renobales, Barron & Kramer, 2013; Bessa *et al.*, 2015; Mapiye *et al.*, 2015). On the contrary, vaccenic acid (11*t*-18:1), is the predominant TFA in meat and milk from forage fed ruminants (Scollan *et al.*, 2014). A potential effect on the protection against CVD by 11*t*-18:1 is having increased attention and is being under debate and intensive research (Gebauer *et al.*, 2011; Tardy *et al.*, 2011; Wang *et al.*, 2012, Wang & Proctor, 2013; Gayet-Boyer *et al.*, 2014). Moreover, this FA is the major precursor for the endogenous synthesis of the health beneficial 9*c*,11*t*-18:2 isomer (rumenic acid) (Palmquist *et al.*, 2004) and about of 20% to 30% of the 11*t*-18:1 absorbed, is converted in 9*c*,11*t*-18:2 in tissues of both ruminants and humans (Turpeinen *et al.*, 2002; Bhattacharya, Banu, Rahman, Causey & Fernandes, 2006; Gruffat, Remond, Durand, Loreau & Bauchart, 2008). For these reasons, the increment in 11*t*-18:1 is currently considered as an important goal to achieve in the improvement of ruminant nutritional value of edible fat (Mapiye *et al.*, 2012, 2015).

Despite the isomer specificity effects of TFA on human health, a global effort in eliminating TFA from human diet, has occurred since 2003, when it was recommended by WHO that TFA intake, should be lower than 1% of total daily energy intake, which corresponds to 2.4 to 3.9 g per day (WHO 2003; FAO, 2010). In order to correspond to this dietary recommendations, several countries have implemented strong regulations involving the reduction by food industry of the partial hydrogenated vegetable oil (iTFA) on food composition and started to be mandatory for pre-packaged foods labeling with information concerning to TFA content (Wang *et al.*, 2012). Moreover, in June 2015 the United States Food and Drug Administration (FDA) determined that *trans* fats derived from the

industrial hidrogenation of vegetable oils are no longer Generally Recognized as Safe (GRAS) and set that iTFA must be removed from all processed food during the next three years (Department of Health and Human Services, 2015; www.fda.gov/consumer, 2015). Also WHO in the " European Food and Nutrition Action Plan 2015-2020" propose a series of measures directed to the food supply chain in order to reduce the intake of SFA, TFA, sugar and salt in the European population. The proposed measures include the development and the implementation of national policies to eliminate *trans* fats from the food supply, with the objective to make the European Region as *trans* fat-free (WHO, 2014). Very recently, on December 2015, the European Commission adopted a report to the European Parliament and to the Council regarding the content of *trans* fats in foods and in the overall diet of the Union population, carring out a preliminary analysis of the potential effectiveness of the measures to be adopted at EU level regarding the reduction of TFA consumption in the European Union (European Commission, 2015).

Thus, with the reduction in the use of iTFA by food industry, the rTFA are gaining relative importance as dietary sources of TFA for humans (Aldai *et al.*, 2013). Enhancing ruminant products with rTFA such as 11*t*-18:1 due to its health positive effects do not match with the recommendations to minimize total dietary TFA (Gebauer *et al.*, 2011).Therefore, it is of indubitable importance enhancing the TFA profile of ruminant's edible fat, decreasing the ratio between the non healthy TFA and the healthy TFA (10*t*-18:1 / 11*t*-18:1ratio). To achieve that objective, a multifactorial approach involving the manipulation of animal diets which results in the increase of 11*t*-18:1 proportion instead that of 10*t*-18:1 in ruminant meats and dairy products is recommended (Mapiye *et al.*,2015).

The conjugated isomers of linoleic acid (CLA) comprise a group of positional and geometrical isomers of linoleic acid (18:2 n-6) characterized by containing conjugated double bonds that can be present in several positional and geometrical configurations (Bessa, Santos-Silva, Ribeiro & Portugal, 2000). Despite of some CLAs presenting the double bonds in *trans* configuration, according to the definition of TFA established by the *Codex Alimentarius,* to support the international regulations in order to reduce TFA from foods, all the CLA isomers are not considered as TFA. Rumenic acid (9*c*,11*t*-18:2) is the main CLA found on ruminant tissues, comprising more than 80% of CLA isomers (Khanal & Dhiman, 2004). Only a minor proportion of 9*c*,11*t*-18:2 content in the tissues is formed by RBH, being about 80% derived from the endogenous conversion of 11*t*-18:1 by Δ9-desaturase (Palmquist *et al.*, 2004). Anti-cancerinogenic, anti-obesity, anti-diabetogenic, anti-atherogenic and anti-inflammatory effects are some of the health benefits attributed to CLA isomers and reported in several reviews (Bhattacharya *et al.*, 2006; Crumb, 2011; Gebauer *et al.*, 2011; Mele *et al.*, 2013; Wang *et al.*, 2012, Wang & Proctor, 2013). However, neither the daily intake of CLA or specifically of 9*c*,11*t*-18:2 were object of the FAO recommendations in 2010, because, at that

time, it was considered that there was not enough sufficient scientific evidence of CLA beneficial health effects. In fact, most of the benefit effects of CLA observed *in vivo* were obtained with animal models, and the results from research in humans are not conclusive. More information is needed to conclude about the isomer specificity of CLA and what is the daily dose needed for the benefits to be obtained (Bhattacharya *et al.*, 2006; Daley, Abbott, Doyle, Nader & Larson, 2010; Crumb, 2011; Gebauer *et al.*, 2011; Castro-Webb, Ruiz-Narvaéz & Campos, 2012; Mele *et al.*, 2013; Wang & Proctor, 2013).

Ruminant´s meat, milk and dairy products, are the main dietary sources of 9*c*,11*t*-18:2 in human diet (Chin, Liu, Storkson, Ha & Pariza, 1992, Bessa *et al.*, 2000; Collomb, Schmid, Sieber, Wechsler & Ryha, 2006; Schmid, Collomb, Sieber & Bee, 2006). However, it constitutes only a small part of total fatty acid content: 3.3 – 23.5 mg/g FA in milk; 2.7 - 6.5 mg/g FA in beef and 8.8 - 19.0 mg/g FA in lamb meat (Collomb *et al.*, 2006; Schmid *et al.*, 2006). The high variation observed for CLA content in ruminant products is due to the influence of several factors, such as production practices (diet/feeding system), animal genetics (individual, gender, breed, species), age, type of tissue and type of muscle and seasonal variations. Among these, the diet/feeding system is the most important one as it provides the substrate for CLA and vaccenic acid synthesis (Collomb *et al.*, 2006; Schmid *et al.*, 2006). Consequently, the human daily intakes of CLA in general and of rumenic acid in particular is highly variable, aggravated with the individual consumption habits of ruminant products. For instance, the intake of 9*c*,11*t*-18:2 can vary from an intake of 140 and 180 mg/day, respectively in Spain and in the United States, to 390 mg/day in Germany (Gebauer *et al.*, 2011).

Polyunsaturated fatty acids

Linoleic (LA; 18:2 n-6; 9*c*,12*c*-18:2) and linolenic acids (ALA; 18:3 n-3; 9*c*,12*c*,15*c*-18:3) (Fig. 1.5) are essential FA, as they only can be achieved by vertebrates thought the dietary intake. Vegetable oils such as sunflower seed oil, soybean oil, corn oil, grape seed oil and cottonseed oil are rich in 18:2 n-6. Vegetable oils from flaxseed, camelina, canola or rapeseed, seeds such as chia seeds, walnuts and green, leafy vegetables have high concentrations of 18:3 n-3 (Gebauer, Psota, Harris & Kris-Etherton,2006; Barceló-Coblijin & Murphy, 2009, Bradbury, 2011). Omega-6 (n-6) and omega-3 (n-3) polyunsaturated fatty acids (n-6 PUFA and n-3 PUFA) are the two FA families of bioactive FA that derive from 18:2 n-6 and 18:3 n-3 respectively. The n-6 FA are defined by having all *cis* methylene interrupted double bonds and by the fact that the last double bond is located at the sixth carbon counting from the FA methyl end, while the n-3 FA have the last double bond located at the third carbon counting from the methyl end. Successive reactions of desaturation and elongation

catalized by ∆-5 and ∆-6 desaturases and respective elongases, synthesize the longer chain PUFA (>C20 chain) (LC-PUFA). For n-6 series which araquidonic acid (AA; 20:4 n-6) is the most relevant FA synthesized from 18:2 n-6. For of n-3 series eicosapentaenoic acid (EPA; 20:5 n-3), docosapentaenoic acid (DPA; 22:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) are the most representative derivatives of 18:3 n-3 (Barceló-Coblijin & Murphy, 2009). The enzymatic complex which is involved in the synthesis of the LC-PUFA is common for both n-3 and n-6 family, occurring competition for the same enzymes by n-3 and n-6 substrates, with preference for 18:3 n-3 in detrimental of 18:2 n-6 (Burdge & Calder 2005; Barceló-Coblijin & Murphy, 2009). This fact influences the proportions of AA, EPA, DPA and DHA synthesized from 18:2 n-6 and 18:3 n-3 respectively, and the balance between the LC-PUFA of the two families that will affect their mechanisms of action in the body.

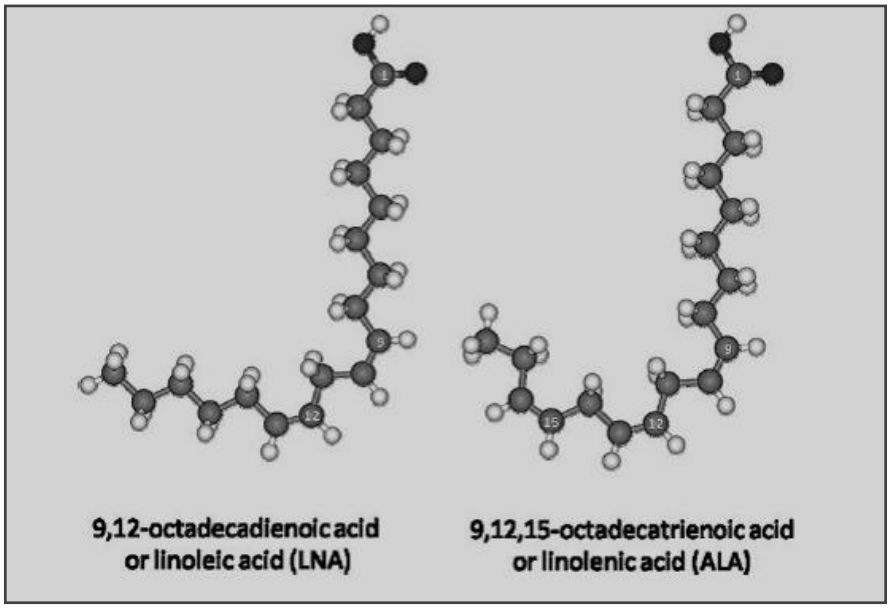


Figure 1.5- Models of the molecular structure of linoleic (or 18:2 n-6) and of linolenic acid (18:3 n-3). Source: Barceló-Coblijin and Murphy (2009)

The physiological mechanisms of action of n-3 and n-6 PUFA leads to different health effects of these two families of FA. The n-6 PUFA are largely linked to the inflammatory processes since their play an important role as precursors of inflammatory mediators. Araquidonic acid is the precursor of inflammatory eicosanoids with high pro-inflammatory potential, such as protaglandin E2 (PGE2) and leukotriene B4 (LTB4) (Calder 2002, 2006, 2010). So, increased AA intake is associated to increased inflammatory responses in humans, what leads to a predisposition, or to an exacerbation of chronic diseases with an inflammatory component, or to a highly inflammatory response in the case of acute inflammation (Calder, 2006; Simopoulos, 2010). In its turn, n-3 PUFA, namely EPA and DHA act in the inflammatory

reaction mostly as anti-inflammatory agents. They can replace the AA incorporation into phospholipids of inflammatory cells, what leads to a less availability of AA to act as substrate for the synthesis of AA-derived eicosanoids and they also inhibit AA metabolism to synthesize the eicosaenoids. In its turn, EPA also gives rise to leukotriene B5 (LTB5), leukotriene E5 (LTE5) and 5-hydroxyeicosapentaenoic acid, that are also eicosaenoids, but with lower pro-inflammatory potential than those synthesized by AA (Calder 2006, 2010). Moreover, EPA and DHA act in the inflammation process as precursors of two families of lipid mediators that were recently described and possess anti-inflammatory and inflammation-resolving actions: the resolvins family, synthesized from both EPA (E-series resolvins) and DHA (D-series resolvins) and the protectins family, synthesized from DHA (Calder, 2010, 2012) (Fig. 1.6).

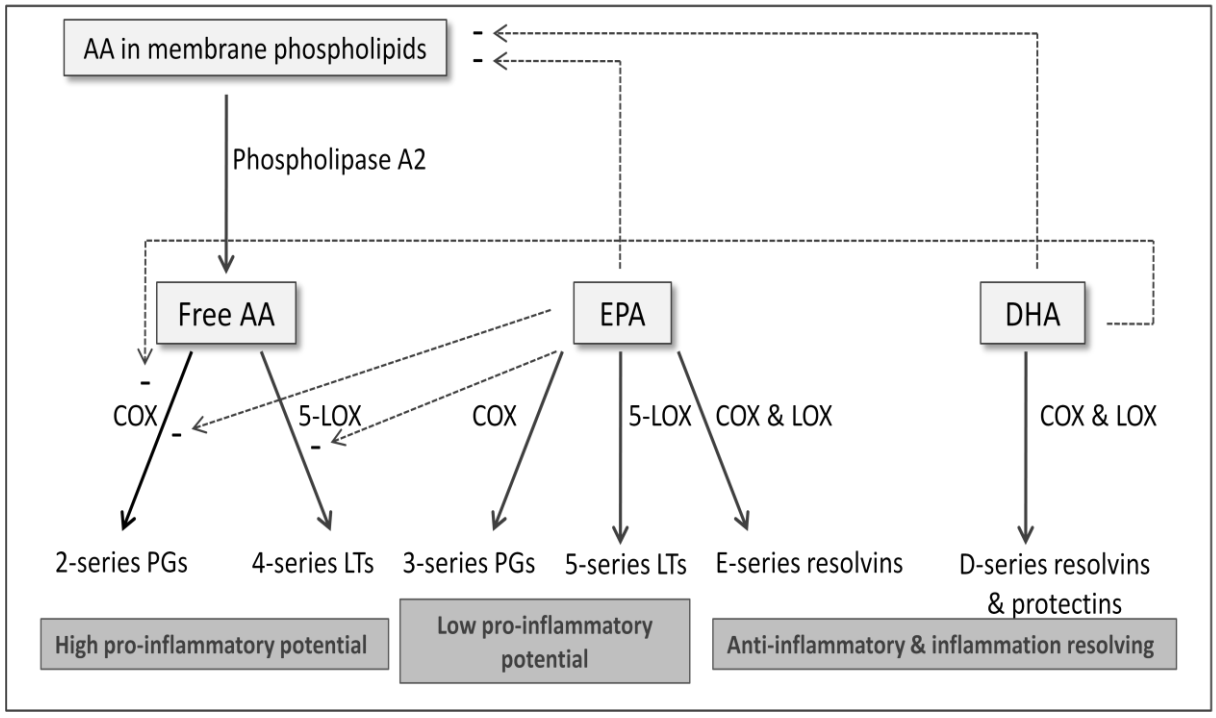


Figure 1.6 - General overview of the anti-inflammatory action of n-3 LC-PUFA, EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) and lipid mediators produced from AA (arachidonic acid), EPA and DHA. COX, cyclooxygenase; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin. Adapted from Calder (2006) and from Calder (2012)

The n-3 LC-PUFA are also related to other anti-inflammatory effects besides the direct alteration of eicosaenoids production. It is the case of the reduction on synthesis of cytokines and of reactive oxygen species and the decrease of the expression of adhesion molecules and of leukocyte chemotaxis (Calder, 2002, 2006). Therefore, due to its anti-inflammatory actions, n-3 LC-PUFA are associated to the decrease of diseases with an inflammatory component such as rheumatoid arthritis, inflammatory bowel diseases, psoriasis, lupus,

asthma, cystic fibrosis, dermatitis among others (Calder, 2012). However, the beneficial health effects of n-3 LC-PUFA are not restricted to their action as regulators of inflammatory processes. Effects, due to other physiological roles of n-3 LC-PUFA, on several other diseases and health disorders are also reported, such in CVD, compromised immunity, obesity, osteoporosis, cancer and neurological diseases as dementia and depression, as it has been reviewed (Calder, 2012). Moreover, due to its high importance as structural component of the cell membrane phospholipids of neuronal synapses in the brain and of photoreceptor cells of retina, a correct DHA intake during embryogenesis, early postnatal stages and childhood is crucial for an optimal cognitive and visual development (Bradbury, 2011; Calder, 2012; Kuratko, Barrett, Nelson & Salem, 2013; Lee, 2013). Although humans are able to desaturate and elongate linolenic acid into *n*-3 LC-PUFA, the conversion has a low efficiency (Burdge & Calder 2005), what have led to the suggestion that EPA and especially DHA, must be considered as conditionally essential FA and their dietary supply needed (Givens & Gibbs, 2008; Calder *et al.*, 2010; Givens, 2010).

The recommended daily intakes of EPA and DHA are rather variable and depend of the country and organization that formulate the dietary recommendations, the target population (age group, gender, healthy or non-healthy individuals) and the beneficial health effects that are suppose to obtain with certain value of intake. Thus, as example, while the recommended daily dose for adults´ intake of EPA plus DHA in US is 270mg/d, in Belgium that value is about 700mg/d (Givens &Gibbs, 2008). However, globally, the daily intake of EPA plus DHA recommended by FAO (2010) is of 250mg/d for adults, of 100-150mg/d for children 2-4 years; of 150-200 mg/d for children 4 – 6 years and of 200-250mg for children 6-10 years. Moreover, for some chronic diseases, especially rheumatoid arthritis, the recommended intakes are around 3g/d of n-3 LC-PUFA. It is also advertised that the recommendations should be personalized according to the estimation of individual FA status and general situation (FAO, 2010). The estimative for the daily intakes of EPA plus DHA shown that a high variation occurs among different regions, being typically low among the western countries (Givens & Gibbs, 2008). In fact, Japan is the country which present the higher daily intake of DHA plus EPA (950mg/d, for adults) in contrast, to Australia (143 mg/d), North America (200mg/d) and Mid Europe (250mg/d) (Givens & Gibbs, 2008). These observations reflect the high relationship between the intake of n-3 LC-PUFA and the populations consumption of seafood, especially that from cold water. Oil-rich fish such as salmon, tuna, sardines, mackerel and herring, since they store the lipids in their flesh, are the better sources of EPA and DHA for the human diet. A single oily fish meal could provide 1.5 – 3.5g of n-3 LC-PUFA (Calder, 2012). Other important source of EPA and DHA is fish oil which is extracted from oily fish flesh or of lean fish liver (as cod), and usually is used as dietary supplement (Givens, 2010; Calder 2012). As alternative to seafood, the meat from

grass/forage fed ruminants can be a valuable source to the dietary intake of n-3 LC-PUFA (Givens *et al.*, 2006; Givens, 2009; Daley *et al.*, 2010; Ponnampalam *et al.*, 2014). This is particularly important in populations where the intake of marine products is very low but the consumption of ruminant meat is high. Docosapentaenoic acid is usually the predominant n-3 LC-PUFA in the meat from grass fed ruminants (Oleksandr, Byelashov, Sinclair & Kaur, 2015) and such as EPA and DHA it possesses many beneficial biological effects, which are mainly relate to the cell membrane functions, the eicosanoid production and the regulation of gene expression (Kaur, Cameron-Smith, Garg & Sinclair, 2011; Oleksandr *et al.*, 2015). Docosapentaenoic acid is an intermediary product between EPA and DHA and it can be readily converted in both EPA and DHA in human tissues as it was recently demonstrated by Miller *et al.* (2013). For that reason Miller *et al.* (2013) suggested that DPA may serve as a storage depot for EPA and DHA in the human body. Meat from grass-fed ruminants represents an important dietary source of DPA in populations such as from Australia and United Kingdom (Givens & Gibbs, 2006; Howe, Meyer, Record & Baghurst, 2006), and in contrast to FAO and many government agencies worldwide which only offer recommendations for EPA and DHA daily intakes, in Australia and New Zealand the guidelines for the n-3 LC-PUFA intake englobe the DPA (National Health and Medical Research Council, 2006).

Maximizing the content of n-3 LC-PUFA is therefore an important production target to improve ruminant's meat nutritional quality and is simultaneously a mean for increasing daily intake of EPA, DPA and DHA without changing the eating patterns of consumers. For several years it was suggested that the n-6:n-3 ratio should be less than 4.0 (Wood *et al.*, 2003). However, actually, and because it is not already demonstrated that the n-6 PUFA intake represents an evident risk for human health and it is considered that the negative health effects associated to a high n-6 : n-3 ratio may be due a lower n-3 PUFA intake, the subject of the dietary guidelines for n-3 PUFA and n-6 PUFA intakes are their absolute amount instead a specific recommendation for n-6 : n-3 ratio (FAO, 2010).

1. 3. Ruminant lipid metabolism

Lipolysis and subsequent biohydrogenation of the dietary PUFA by rumen microorganisms (RBH) are the reason why the fat of ruminants contains a much higher level of SFA and less PUFA than those present in the animal diet, which is regarded as prejudicial to human health (Doreau & Chilliard, 1997; Bauman, Baumgard, Corl & Griinari, 1999; Shingfield, Bonnet & Scollan, 2013). However, the production of FA with beneficial health effects such as 9*c*,11*t*-18:2 and 11*t*-18:1 found in the ruminant products depends of the RBH occurrence and extension. In other hand, the RBH of dietary PUFA is not total and there is a proportion of

PUFA that flows from the rumen and is absorbed in the duodenum and deposited in the tissues, where it can undergo to desaturation and elongation, to form LC-PUFA (Figure 1.7).

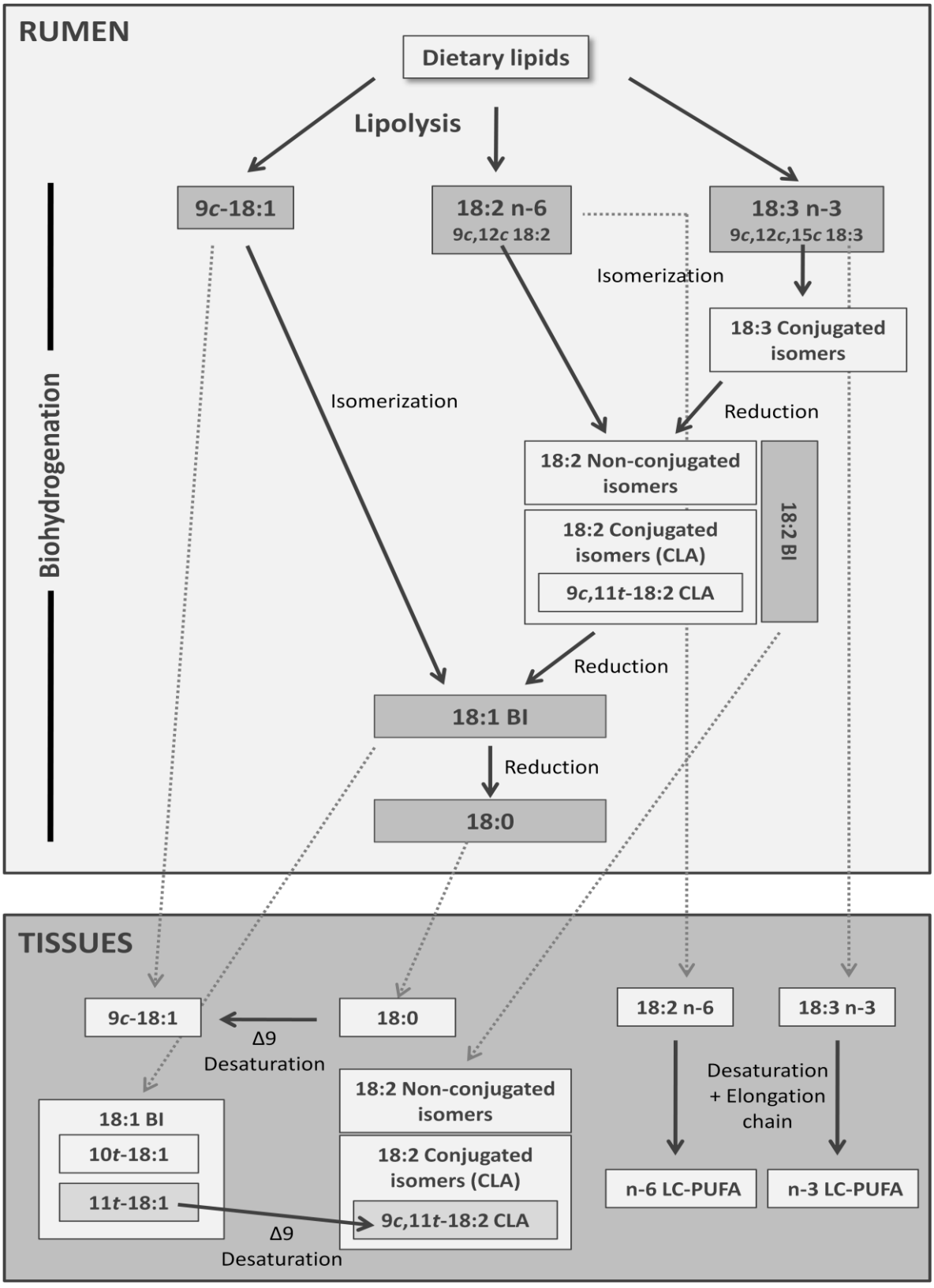


Figure 1.7 – Global overview of the main pathways of rumen lipid metabolism and relationship with the deposition and conversion of bioactive FA in tissues. BI – Biohydrogenation intermediates

1.3.1. Rumen lipid metabolism: Lipolysis and Biohydrogenation

Lipolysis and Biohydrogenation pathways

Ruminant diets typically contain between 20 and 40g lipid/kg dry matter (Shingfield, Bernard, Leroux & Chilliard, 2010) depending their lipid profile on the diet composition. Grass and forages, due to their largely content in glycolipids and phospholipids, present high levels of linolenic (18:3 n-3) and linoleic acid (18:2 n-6), while concentrates, due to grains and seed oils used in their formulation, are rich in 18:2 n-6 and oleic acid (9*c*-18:1) (Bauman *et al.*, 1999; Buccioni, Decandia, Minieri, Molle & Cabiddu, 2012). Dietary unsaturated fatty acids (UFA) are considered as toxic to rumen microorganisms and RBH might be a protection mechanism of animal health (Maia, Chaudhary, Figueres & Wallace, 2007; Jenkins, Wallace, Moate & Mosley, 2008; Lourenço, Ramos-Morales & Wallace, 2010). Ruminal biohidrogenation may also act as a mechanism to rumen microorganisms synthesise *trans* FA which are incorporated into their cellular membranes, increasing membranes stability and thus, protecting microbes when they are subject to physiological stress induced by environmental factors such as high concentrations of PUFA (Bessa *et al.*, 2000, Bessa *et al.*, 2015).

After ingestion the first step of lipid digestion is the hydrolysis of the ester bonds of the dietary lipids by the microbial lipases, process in which a carboxyl group is free, what it is a pre-requesite to the beginning of BH (Sinclair, 2007). Lipolysis is a very rapid process, however, in certain conditions, as in the presence of antibiotics or low rumen pH, its extension can be decreased (Doreau & Chilliard, 1997). Few ruminal microorganisms possess lipases which are capable to hydrolyze the ester bond (Buccioni *et al.*, 2012). Within the rumen microorganisms, lipolysis is mainly restricted to bacteria. Protozoa also possess lipase activity, but it is not very consistent and fungi do not hydrolyse dietary lipids (Lourenço *et al.*, 2010; Buccioni *et al.*, 2012). The hydrolysis of ester linkages by microbial lipases is a very specific activity. For instance, *Butyrivibrio* spp. and *Anaerovibrio lipolytica* both possess lipase activity, but while *Butyrivibrio* like species are capable to hydrolyze the phosphor- and galactolipids, *Anaerovibrio lipolytica* can hydrolyze triacylglycerols. For that reason, *Anaerovibrio lipolytica* is more associated to lipase activity in animals fed mainly with concentrates and *Butyrivibrio* spp. in animals fed with mainly with forages (Buccioni *et al.*, 2012).

After lipolysis, the free fatty acids (FFA) are subjected to RBH (Sinclair, 2007). This is a multi-step process, involving the sequential actions of two classes of microbial enzymes, isomerases and reductases (Jenkins *et al.*, 2008). Several studies were developed in the last years concerning the lipid metabolism in the rumen, including the characterization of the

metabolic pathways of RBH, the numerous biohydrogenation intermediates (BI) which are formed and the microbial species involved (Jenkins *et al.*, 2008; Lourenço *et al.*, 2010, Shingfield *et al.*, 2010, 2013; Buccioni *et al.*, 2012). After an initial isomerization step, RBH of C18 FFA proceeds with the saturation of the double bonds of 9*c*-18:1, 18:2 n-6 and 18:3 n-3, which culminates in the formation of stearic acid (18:0), as the final product and in the generation of several BI during the intermediate metabolic steps. Generally, between 58 to 87% of dietary oleic acid is biohydrogenated while for 18:2 n-6 and 18:3 n-3 the proportions range from 70-96% and 85-100%, respectively (Shingfield *et al.*, 2013).

Few studies have deeply analyzed the RBH of 9*c*-18:1 (Figure 1.8). Classically, 18:0 has been considered as the unique product of 9*c*,18:1 RBH, but *in vitro* results from recent studies shown that it also occurs the formation of BI such as oxygenated FA (10OH 18:0 and 10O 18:0) and several *trans*-18:1 isomers with double bonds from C6 through C16 (Jenkins *et al.*, 2008; Shingfield *et al.*, 2010).

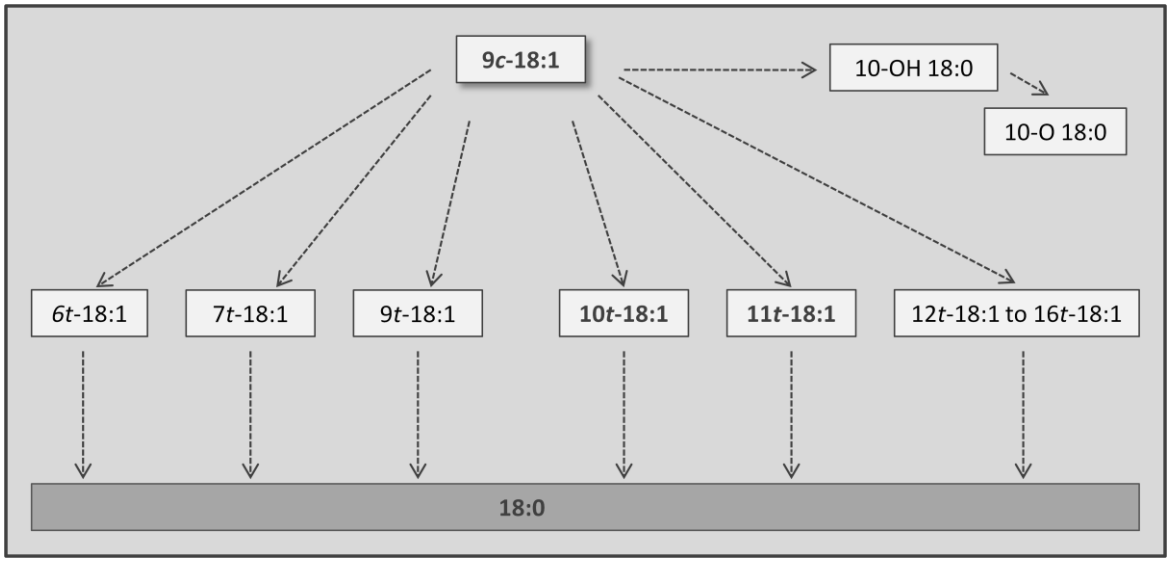


Figure 1.8- Biohydrogenation of oleic acid (9*c*-18:1). Adapted from Shingfield *et al.* (2010).

For 18:2 n-6 and 18:3 n-3, the initial isomerization involves the *cis*-12 double bond and results in the formation of conjugated isomers of the linoleic acid (CLA) and of linolenic acid (Figure 1.9 and Figure 1.10) (Shingfield *et al.*, 2010). In the case of 18:2 n-6, besides CLA synthesis, it is also produced a non-conjugated diene isomer (9*t*,12*c*-18:2) and oxygenated FA (9c,13OH 18:1, 13OH 18:0 and 13O 18:0). Then, a first reduction occurs, producing 18:1 BI, which are mainly *trans*-18:1isomers. The final metabolic step is the reduction of 18:1 BI to 18:0 (Jenkins *et al.*, 2008; Shingfield *et al.*, 2010). As the conversion of the diene isomers to monoenes occurs rapidly and their reduction to 18:0 is low, the last step of RBH is

considered as rate-limiting, leading to an accumulation of 18:1 BI in the rumen. Consequently, a higher content of 18:1 BI becomes available for absorption and deposition in tissues than of 18:2 BI (Bauman *et al.*, 1999). As 18:3 n-3 presents three double bonds, the metabolic pathways responsible for its RBH are more complex than that of 9*c*-18:1 and 18:2 n-6 (Shingfield & Wallace, 2014). After the first isomerization, which origins the conjugated triene isomers, there are three sequential reductions. The first one yields non-conjugated diene isomers and CLAs, the second reduction results in 18:1 BI and the third in 18:0 (Shingfield *et al.*, 2010). For many years, research in RBH has maintained the focus on the major metabolic pathways and BI, leading to the persistence of many oversimplified pathways (Jenkins *et al.*, 2008). It was the case of the formation of CLAs as BI of 18:3 n-3 RBH. Due to the simplification of RHB metabolic pathways, during years, it was considered that the hydrogenation process of 18:3 n-3 do not lead to the formation of CLA isomers (Jenkins *et al.*, 2008). Destaillats, Trottier, Galvez and Angers (2005) suggested the production of two CLA isomers, 9*c*,11*t*-18:2 and 13*t*,15*c*-18:2, through 18:3 n-3 RBH, but the first report that 9*c*,11*t*-18:2 may effectively be formed from RBH of linolenic acid only occurred in 2011 by Lee and Jenkins, with an *in vitro* study using a stable isotope tracer to investigate the BH of ^{13}C-linolenic acid. The authors reported the production of eight intermediate conjugated diene isomers from linolenic BH, including 9*c*,11*t*-18:2, concluding that the 18:3 n-3 RBH pathways can be even more complex than that what it was proposed by Shingfield *et al.* (2010) (Figure 1.10). Moreover, also 18:2 non-conjugated BI from 18:3 n-3 RBH have been identified in the digestive content and ruminant fat, showing the complexity of RBH pathways of 18:3 n-3 and the high diversity of intermediate products. It is the case of 12*c*,15*c*-18:2, which it was identified in the intramuscular fat of lambs fed dehydrated lucerne and 6% linseed oil (Alves & Bessa, 2007; Bessa *et al.*, 2007) and of 10*t*,15*c*-18:2, that was identified in 2014 by Alves and Bessa, in the digestive content and in meat of lambs fed with a complete diet composed by concentrate and dehydrated lucern (1:1) and supplemented with 8% of a vegetable oil blend (soybean plus linseed oil 1:2 vol/vol).

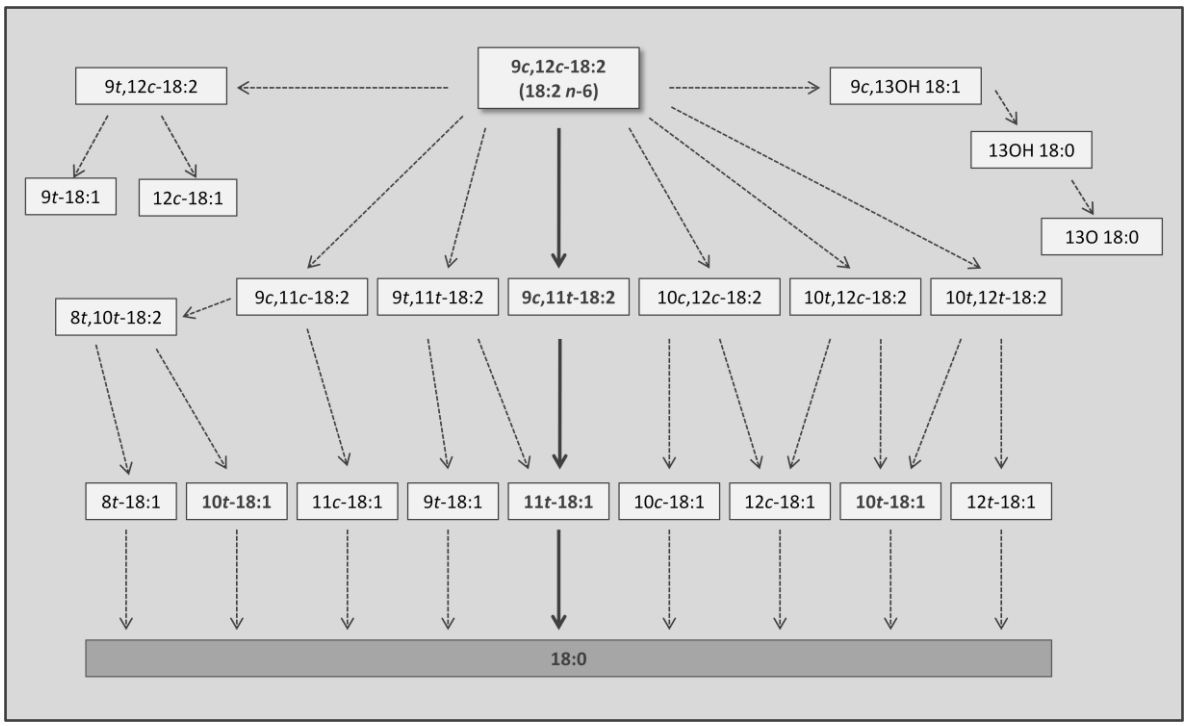


Figure 1.9 -Biohydrogenation of linoleic acid. Arrows with solid lines highlight the major pathway and arrows with dashed lines highlight minor metabolic pathways. Adapted from Shingfield *et al.* (2010).

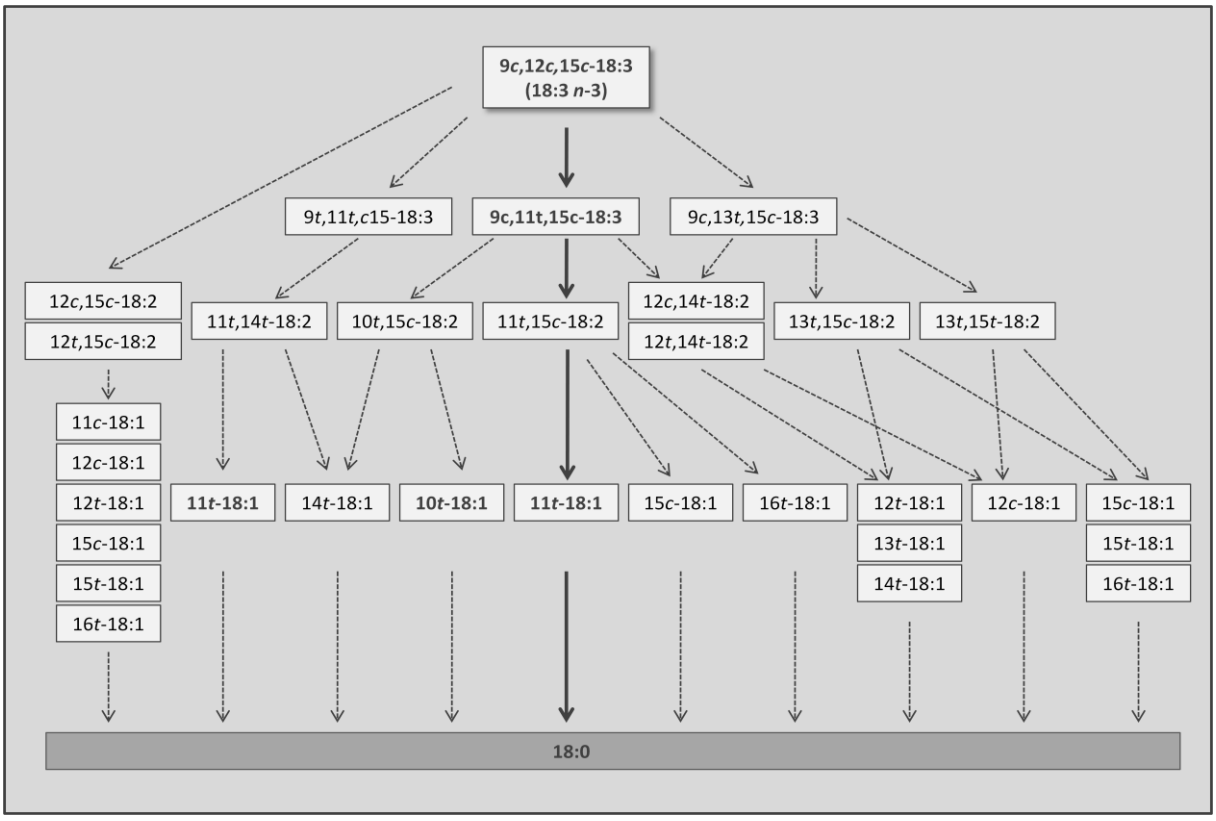


Figure 1.10 - Biohydrogenation of linolenic acid. Arrows with solid lines highlight the major pathway and arrows with dashed lines highlight minor metabolic pathways. Adapted from Shingfield *et al.* (2010).

The role of rumen microorganisms in biohydrogenation

The population of rumen microorganisms is mainly composed by ciliate protozoa, anaerobic bacteria, archaea and anaerobic fungi. Biohydrogenation of dietary PUFA is mainly performed by rumen bacteria, especially those with cellulolytic activity (Jenkins *et al.*, 2008). Simplistically, bacteria involved in the RBH process can be classified into two functional groups: the group A bacteria which hydrogenates linoleic and linolenic acids to 18:1 BI whereas the group B bacteria perform the last step of the BH, forming stearic acid from the reduction of 18:1 BI (Kemp, White & Lander, 1975; Harfoot & Hazlewood, 1997). *Butyrivibrio fibrisolvens* is a cellulolytic bacteria and it is the most important rumen microorganism involved in RBH (Jenkins *et al.*, 2008; Lourenço *et al.*, 2010). *Butyrivibrio fibrisolvens* belongs to the group A bacteria, and its action stops in the 18:1 BI formation. For the last metabolic step of RBH is necessary the action of bacteria from group B such as *Butyrivibrio proteoclasticum* (previsously designated by *Fusocillus spp.* (Kemp *et al.*, 1975; Lourenço *et al.*, 2010).However, Van de Vossenberg and Joblin (2003) have isolated a strain of *Butyrivibrio hungatei* from bovine rumen which can reduce by itself, C18 PUFA to stearic acid. Therefore, the suppression of *Butyrivibrio proteoclasticum and Butyrivibrio hungatei,* or depress of their activity, is a target for many strategies to improve FA profile of ruminant's products (Jenkins *et al.*, 2008).

Although up of half of the rumen biomass can be from protozoa origin (Williams & Coleman, 1992) and the role of ciliate protozoa in RBH is not totally understood, it has been generally considered that ciliate protozoa are not directly related to RBH (Jenkins *et al.*, 2008). Protozoa lipids contain proportionally higher levels of PUFA than bacteria (Harfoot & Hazlewood, 1997; Devillard, McIntosh, Newbold & Wallace, 2006) and their membranes present a high content in 11*t*-18:1 and 9*c,*11*t*-18:2 (Devillard *et al.*, 2006), meaning that protozoa may represent an important reservoir of those FA. The mechanisms by which rumen protozoa incorporate those high levels of PUFA, 11*t*-18:1 and 9*c,*11*t*-18:2 are not clear. The ingestion of chloroplasts, which are rich in PUFA may justify the high levels on those FA in protozoa composition (Huws *et al.*, 2012). However, that is not an explanation for their high content in vaccenic and rumenic acid, as those FA are products of RBH. It was reported that ciliate protozoa may isomerize linoleic acid into 9*c*,11*t*-18:2 and other CLA isomers but they do not seems to produce 18:1 BI and 18:0 (Or-Rashid, AlZahal & McBride, 2008; Or-Rashid, AlZahal & McBride, 2011). In other way, apparently rumen protozoa do not synthesize vaccenic and rumenic acid from desaturation of 18:0 (Devillard *et al.*, 2006). Other possible hypothesis to explain the concentration of 11*t*-18:1 and 9*c*,11*t*-18:2 in protozoa is the incorporation of 11*t*-18:1 and 9*c*,11*t*-18:2 produced by ingested bacteria (Jenkins *et al.*, 2008) or the establishment of symbiotic relationship with biohydrogenating bacteria (Boeckaert *et al.*, 2009). Nevertheless, the retention of high levels of PUFA, 11*t*-18:1

and 9*c*,11*t*-18:2, that may escape to a total BH by ciliate protozoa has implications on the flow of those FA from the rumen and their availability for absorption in duodenum and deposition in the animal tissues. A selective retention of protozoa within the rumen has been reported (Dehority, 2003; Huws *et al.*, 2012), affecting the availability for absorption of the PUFA. Elucidation on the role of ciliate protozoa in RBH and their relationships with biohydrogenating bacteria, the incorporation of 11*t*-18:1 and 9*c*,11*t*-18:2, and mostly, the selective retention of rumen protozoa with consequences to absorption and deposition in meat and milk of beneficial FA, is important to support the strategies to improve nutritional value the products from ruminants through the manipulation of RBH.

Only a minor part of the biomass of ruminal microorganisms is composed by anaerobic fungi. Some ruminal fungi, as *Neocallimastix frontalis* may synthesize 9*c*,11*t*-18:2 from linoleic acid, however, this activity is very small comparing to that of *B. fibrisolvens* (Maia *et al.*, 2007). Also Nam and Garnsworthy (2007) have reported that rumen fungi may hydrogenate linoleic acid to 11*t*-18:1 as the end product, but with a lower BH rate than that of rumen bacteria. As far as we know no information is available about the eventual participation of archaea on RBH.

Factors affecting lipolysis and biohydrogenation

In general, factors that influence the rumen environment also affect the lipid metabolism and deposition in meat. The nutritional factors are the most important, since they can directly and rapidly interfere with the balance between species of rumen microbial community and thus, with the metabolic pathways of BH. It is well known that the feeding system and the composition of the diets, including the forage:concentrate ratio, influences the fatty acid composition of meat (Fisher *et al.*, 2000; Santos-Silva *et al.*, 2002a; Bessa *et al.*, 2005; Bessa *et al.*, 2008; Alfaia *et al.*, 2009; Rosa *et al.*, 2014), as well as the quantity and composition of the dietary lipid supplements (Scollan *et al.*, 2001; Bolte, Hess, Means, Moss & Rule, 2002; Wachira *et al.*, 2002; Santos-Silva, Bessa & Mendes, 2003; Cooper *et al.*, 2004; Demirel *et al.*, 2004; Santos-Silva, Mendes, Portugal & Bessa, 2004; Bessa *et al.*, 2005; Boles, Kott, Hatfield, Bergman & Flynn, 2005; Bessa *et al.*, 2007; Jerónimo *et al.*, 2009; Manso, Bodas, Castro, Jimeno & Mantecon, 2009; Radunz *et al.*, 2009; Ferreira *et al.*, 2014). More recently, it has been reported that dietary plant secondary compounds, such as condensed tannins (CT) also may influence RBH pathways and consequently, meat lipid composition (Vasta *et al.*, 2009b; Jerónimo *et al.*, 2010; Whitney, Lupton & Smith, 2011; Buccioni *et al.*, 2012).

In other way, the rumen microbiota diversity and ecological balance may also be influenced by intrinsic factors related to the animal genetics (species, individual variability) (Williams & Coleman,1992) which may interfer with the rumen lipid metabolism.

The occurrence of trans-10 shift

It was considered for many years that 11*t*-18:1 was the main 18:1 BI. However, due to the improvement of the analytical methods, boosted by the intensive research on BH pathways it is actually recognized that 11*t*-18:1 is not always the major 18:1 BI produced during the BH of oleic, linoleic and linolenic acids, and that under certain rumen environmental conditions it is replaced by the 10*t*-18:1 (*trans*-10 shift) (Griinari & Bauman, 1999). Avoiding the *trans*-10 shift is an important goal in the development of strategies to improve ruminants meat nutritional value, as it may have direct negative effects in human health (Aldai *et al.*, 2013) and also because 10*t*-18:1 is not a precursor of 9*c*,11*t*-18:2 in tissues (Bessa *et al.*, 2015). Moreover, if rumen conditions favour's the occurrence of the metabolic pathways related to the synthesis of 18:2 BI with the 10*t*- double bond, mostly 10*t*,12*c*-18:2, which reduction results in 10*t*-18:1, the pathways that result in 9*c*,11*t*-18:2 and in 11*t*-18:1 may be compromised.

The type of the carbohydrates present in the diet is the most important factor affecting the biohydrogenation pathways, being directly related to the occurrence of the *trans*-10 shift (Bessa *et al.*, 2015). It is well established that in ruminants fed with forage based diets, the main 18:1 *trans* isomer present in meat and milk is 11*t*-18:1. However, when diets present a low fiber content and are rich in sugars and/or starch, such as in the concentrate based diets, the dominant pathway of BH conducts to the formation of 10*t*,12*c*-18:2, leading to high levels of 10*t*-18:1 in meat and milk (Griinari *et al.*, 1998; Bessa *et al.*, 2005; Shingfield & Griinari, 2007; Alfaia *et al.*, 2009; Mapiye *et al.*, 2012; Rosa *et al.*, 2014).Occurrence of the *trans*-10 shift is supposed to be as consequence of the alterations in rumen microbial populations due to the starch fermentation and the decrease of ruminal pH. The RBH is strongly affected by the pH (Bessa *et al.*, 2000) and a decrease in rumen pH can result in a shift on the bacterial population (Jenkins *et al.*, 2008). The activity of cellulolitic bacteria such as *B. fibrisolvens* is the major responsible by the lipolysis and it is associated to the main pathways of RBH, and to high levels of 11*t*-18:1 and 9*c*,11*t*-18:2 synthesis (Jenkins *et al.*, 2008). However, these bacteria are very sensitive to low pH. Martin and Jenkins (2002) suggested that, to maximize 9*c*,11*t*-18:2 synthesis in the rumen, pH has to be maintained above 6.0 because of the sensitivity of cellulolitic bacteria to acidic condition. *Propionibacterium*, *Streptococcus*, and *Lactobacillus* are more numerous in the rumen with concentrate diets and are associated with the production of 10*t*,12*c*-18:2 (Jenkins *et al.*, 2008). Therefore, when starchy diets are

used for ruminants, the activity of amilolytic bacteria may be more expressive than that of cellulolytic bacteria and the alternative pathway for the formation of 10*t*,12*c*-18:2 and 10*t*-18:1 becomes dominant.

1.3.2. Post absorptive metabolism of unsaturated fatty acids on intramuscular fat

When reach to duodenum, FA are mainly adsorved on feed particles, bacteria and desquamated cells. For the absorption by epitelial cells of intestinal mucosa, could be possible, FA are solubilised in the aqueous environment of the intestinal lumen through the formation of micelles due to the action of biliary salts and lysolecithins. After being absorved by jejunum enterocytes, FA are re-esterified in triacylglycerols (TAG) and phospholipids, which are then transported into the lymphatic system by lipoproteins such as chylomicrons and very low density lipoproteins (VLDL) and after, into the circulatory system through the low density lipoproteins (LDL) and high density lipoproteins (HDL) (Doreau & Chilliard, 1997; Demeyer & Doreau, 1999). Chylomicrons and VLDL incorpore mainly TAG, while phospholipids, cholesterol and cholesterol esters are mostly incorporated in LDL and HDL (Bauchart, 1993; Demeyer & Doreau, 1999).

Lipid composition of ruminant's intramuscular fat

Intramuscular fat comprises the FA present in the adipocytes which are deposited isolated or aggregated between the muscular fibers and those FA which compose the cellular lipids of muscular cells (membranes phospholipids, cholesterol and cytosolic droplets of TAG) (Demeyer & Doreau, 1999; Raes *et al.*, 2004). Intramuscular adipocytes contain the majority of TAG of muscle and are associated to the neutral lipids fraction (NL) of the IMF. Muscular fibers, due to their high proportion in phospholipids, are mainly associated to the polar lipids fraction (PL) of IMF (Raes *et al.*, 2004). Comprising its PL and NL fractions, IMF content, in general, can range between 1 and 5 g/100g muscle, containing 45 to 48, 35 to 45 and up of 5.0 g/100g FA as SFA, MUFA and PUFA, respectively (Scollan *et al.*, 2006). However, the neutral fraction of IMF is characterized by a high proportion of SFA and MUFA (Raes *et al.*, 2004) and by the preferential deposition of BI such as 9*c*,11*t*-18:2 relatively to PL (Jerónimo *et al.*, 2011). The PL fraction shows a high proportion of PUFA comparatively to NL, comprising the main proportion of n-3 and n-6 LC-PUFA (Scollan *et al.*, 2006; Wood *et al.*, 2008, Bessa *et al.,* 2015). However, when IMF content is high, the contribution of the NL increases and consequently reduces the proportion of PUFA from PL in total of FA content (Wood *et al.*, 2008). Due to the functions of phospholipids as membrane cells constituents and in order to maintain membrane properties and cell integrity, PUFA deposition in PL is

under strictly regulatory control and thus, is less influenced by dietary factors than in NL (Raes *et al.*, 2004, Scollan *et al.*, 2006). Thus, dietary manipulation of FA composition of IMF will be mainly reflected in NL than it is in the PL fraction.

Synthesis of fatty acids in muscle

The FA deposited in intramuscular lipid may be obtained directly from the diet or may be synthesized *de novo* using the final products of the energy metabolism in rumen. Diet-derived FA includes the RBH products (intermediate (BI) and final (18:0)) and those dietary PUFA that escape from RBH. These FA can be directly deposited in the intramuscular adipocites and muscular fibers or to be submited to elongation and/or desaturation before deposition. The FA from the *de novo* synthesis mainly results from non lipid precursors, mostly acetate and to a lesser extent, lactate (Demeyer & Doreau 1999; Shingfield *et al.*, 2013). The main final product of *de novo* FA synthesis is 16:0 which can be used as substract for further elongation or desaturation (Shingfield *et al.*, 2013). Synthesis of FA up to 16:0 occurs in the cytoplasm from acetyl-CoA and β-hydroxybutyrate that are derived from mitochondrial oxidation.Elongation of 16:0 to longer FA up to C22 occurs in the mitochondria while elongation and desaturation of FA ≥ C18 occurs in the microsomes (Demeyer & Doreau, 1999). Fatty acids elongases present a specific affinity for FA with different saturation degrees: while SFA and MUFA act as substracts for elongases 1,3 and 6, PUFA act as substracts for elongases 2,4 and 5 (Cherfaoui *et al.*, 2012). Desaturation of FA is performed by Δ5, Δ6 and Δ9-desaturases (Shingfield *et al.*, 2013). The first two enzymes are linked to the synthesis of n-3 and n-6 LC-PUFA from the dietary n-3 and n-6 PUFA which escape from RBH whereas Δ9-desaturase is responsible by the convertion of SFA to their respective MUFA and of 11*t*-18:1 to 9*c*,11*t*-18:2.

Endogenous synthesis of rumenic acid in muscle

The content of 9*c*,11*t*-18:2 found in ruminant IMF depends of the rumen production of both 9*c*,11*t*-18:2 and 11*t*-18:1 and of the endogenous synthesis by the activity of ∆9-desaturase on 11*t*-18:1. This last process is the primary source of 9*c*,11*t*-18:2 in tissues (Bauman, 1999; Griinari & Bauman, 1999) (Figure 1.11). Palmquist *et al.* (2004) have estimated that about 28% of the 11*t*-18:1 produced in rumen originated, by endogenous desaturation, 87% of the 9*c*,11*t*-18:2 deposited in the *longissimus* muscle of lambs. Moreover, in the same study, the authors also reported that the endogenous synthesis of 9*c*,11*t*-18:2, on adipose tissue and muscle, was negatively correlated to its RHB supply (r=-0.80). From that model the authors have inferred that when the synthesis of 9*c*,11*t*-18:2 in rumen is low and of 11*t*-18:1 is high, the proportion of 9*c*,11*t*-18:2 endogenously produced increases, being reduced when the

synthesis of 9*c*,11*t*-18:2 is high and that of 11*t*-18:1 is low. Althought this relationship translates the effects of the diet composition on RBH and consequently on the proportions of 9*c*,11*t*-18:2 and 11*t*-18:1 available to be absorbed in duodenum and incorporated in tissues, as far as we known, it is still not confirmed *in vivo*.

In high-forage diets, the extensive RBH leads to a high availability for absorption of 11*t*-18:1, which will act as subtract to endogenous synthesis of 9*c*,11*t*-18:2. Moreover, as forage is richer in 18:3 n-3 than in 18:2 n-6, less 9*c*,11*t*-18:2 results from RBH, constraining its availability for rumen outflow and direct incorporation in tissues. Thus, in this case, the predominant pathway for 9*c*,11*t*-18:2 deposition in tissues it will be it's endogenous synthesis from 11*t*-18:1 desaturation. If the diet contains a higher content of 18:2 n-6, as in the case of high-concentrate diets or dietary supplementation with oils rich in 18:2 n-6, if the *trans*-10 shift does not occur, the proportion of 9*c*,11*t*-18:2 produced by 18:2 n-6 biohydrogenation increases, enhancing its availability and deposition in tissues and the relative importance of that pathway to the total 9*c*,11*t*-18:2.

Recently, it was reported that 9*t*-16:1 also may act as substract for the endogenous synthesis of 9*c*,11*t*-18:2 in bovine adipocytes. This pathway involves the elongation of 9*t*-16:1 to 11*t*-18:1 by the fatty acids elongases 5 (*ELOVL5*) and 6 (*ELOVL6*) and then the desaturation of 11*t*-18:1 to 9*c*,11*t*-18:2 by Δ9-desaturase (Kadegouda, Burns, Miller & Duckett, 2013a).

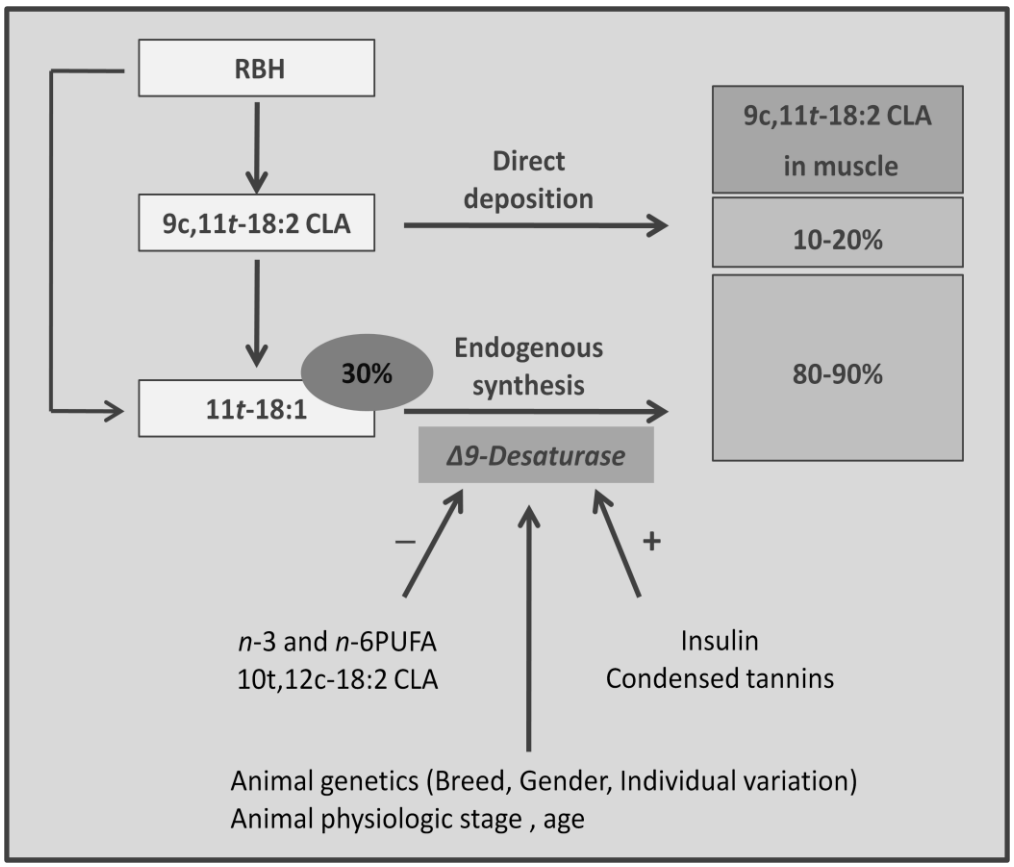


Figure 1.11- Relationship of the 9*c*,11*t*-18:2 content in muscle with its supply via rumen biohidrogenation (RBH) synthesis and via 11*t*-18:1 endogenous conversion by ∆9-desaturase. Based on data from Palmquist *et al.* (2004).

The enzyme Δ9-desaturase is an endoplasmatic reticulum enzyme with 359 amino acid residues, which catalyses the desaturation of 10- to 19- carbon fatty acyl-CoA substrates resulting in the introduction of a *cis* double bond between the carbons 9 and 10 of the fatty acid moiety (Ntambi, 1999). The desaturation of the fatty acyl-CoA substrates results from the conjunction of Δ9-desaturase with NADH(P)-cytochrome *b*5 reductase, the electron acceptor cytochrome *b*5 and molecular oxygen (Paton & Ntambi, 2009). Several saturated (14:0, 16:0, 17:0, 18:0) and *trans*-monounsaturated (7*t*-18:1, 11*t*-18:1 and, presumably 13*t*-, 14*t*- and 15*t*-18:1) fatty acyl-CoA, may be desaturated by the activity of Δ9-desaturase, resulting in *cis*-9 MUFA and in some 9*cis*,x*trans* dienes (Bessa *et al.*, 2015). However, as the preferred subtracts for Δ9-desaturase activity are the stearoyl- and palmitoyl-CoA, which conversion origins oleoyl- and palmitoleoyl-CoA respectively, originating the major products of Δ9-desaturase in mammals, oleic (9*c*-18:1) and palmitoleic (9*c*-16:1) acids (Ntambi, 1999; Paton & Ntambi, 2009) Nevertheless, due to the variability of substrates and products, Δ9-desaturase activity affects the lipid composition of phospholipids, mainly by increasing the availability of 9*c*-18:1, and of triglycerides, mainly through the synthesis of CLAs such 9*c*,11*t*-18:2 and 9*c*,7*t*-18:2.

The Δ9-desaturase is codified by the *SCD* gene. Homologues of *SCD* gene have been identified and characterized in several mammalian species, including rats, mice, hamsters, pigs, sheep, cows, goats and humans (Ntambi 1999; Paton & Ntambi, 2009; Shingfield & Wallace, 2014). In mice, four isoforms (*SCD*-1, *SCD*-2, *SCD*-3, *SCD*-4) are identified, but ruminants, as well as humans, express two isoforms of *SCD* gene (*SCD*-1 and *SCD*-5) (Lengi & Corl, 2007; Paton & Ntambi, 2009; Shingfield & Wallace, 2014). The expression of *SCD*-1 gene is higher in fat tissue than it is in muscle and in growing ruminants the adipose tissue is considered as the major site of Δ9-desaturase activity (Griinari & Bauman, 1999; Shingfield & Wallace, 2014). Although the *SCD*-5 isoform be expressed in several tissues, its contribution to the desaturation of FA in ruminants remains unclear (Shingfield *&* Wallace, 2014). In cattle, *SCD*-5 is mostly expressed in the brain (Lengi & Corl, 2007). In lactating goats were observed higher *SCD*-5 mRNA levels in mammary gland than in liver or adipose tissues (Toral, Chilliard & Bernard, 2012).

As endogenous synthesis of 9*c*,11*t*-18:2 depends on Δ9-desaturase activity, factors that regulate *SCD* gene expression and/or enzyme expression and activity, also control the level of 9*c*,11*t*-18:2 on tissues. The *SCD*-1 gene expression is regulated by several factors such as dietary, hormonal and genetics, which may act as regulators of transcription and/or at the *post*-transcriptional level, in the mRNA stability (Ntambi, 1999; Paton & Ntambi, 2009; Shingfield & Wallace, 2014; Bessa *et al.*, 2015). Feeding system is the main regulator of *SCD*-1 gene expression (Dervishi *et al.*, 2010). High-carbohydrate diets increase *SCD*-1 gene expression (Paton & Ntambi, 2009). However, in spite of glucose and fructose may activate by themselves the *SCD*-1 transcription (Bessa *et al.*, 2015), it is considered that much of the reported increase in *SCD*-1 expression in the presence of starch-rich diets is due to the direct influence of insulin, which increases the activation of the sterol regulatory element binding protein-1c (SREBP-1c) and consequently, the activation of the *SCD*-1 gene promoter (Paton & Ntambi, 2009; Bessa *et al.*, 2015). Lipid composition of the diet also regulates the expression of the *SCD*-1 gene in adipose tissue of ruminants (Hausman *et al.*, 2009). Dietary PUFA, particularly 18:3 n-3, are associated to the reduction of *SCD*-1 transcription, of *SCD*-1 mRNA stability and of Δ9-desaturase expression and activity (Ntambi, 1999; Daniel *et al.*, 2004; Waters, Kelly, O'Boyle, Moloney & Kenny, 2009; Dervishi, 2010; Corazzin, Bovolenta, Sacca, Bianchi & Piasentier, 2013; Shingfield & Wallace, 2014). Also 10*t*,12*c*-18:2 is being implicated in down-regulation of *SCD* gene expression (Lee, Pariza & Ntambi, 1998; Baumgard, Matitashvili, Corl, Dwye & Bauman, 2002; Gervais, McFadden, Lengi, Corl & Chouinard, 2009, Lengi & Corl, 2010) and in Δ9-desaturase activity (Wynn *et al.*, 2006; Kadegouda, Burns, Pratt & Duckett, 2013b). By contrast, it seems that dietary plant secondary compounds, such as condensed tannins up-regulate Δ9-desaturase expression (Vasta *et al.*, 2009c; Rana, Tyagi, Hossain & Tyagi, 2012). Although the

regulation of *SCD* gene expression and Δ9-desaturase activity by genetics is lower than it is by nutrition, differences between breeds have been identified (Sinclair, 2007; Smith *et al.*, 2009; Costa *et al.*, 2013a, 2013b; Shingfield *et al.*, 2013), as well as a high individual variation in the *SCD* mRNA levels (Costa *et al.*, 2013a; 2013b) and in Δ9-desaturase protein expression (Herdmann, Nuernberg, Martin, Nuernberg & Doran, 2010). These findings alert that genetic variability must be taken into account in the development of nutritional strategies that involve the manipulation of Δ9-desaturase metabolism to enhance the concentration of 9*c*,11*t*-18:2 on ruminant meat though the 11*t*-18:1 endogenous conversion.

Synthesis of LC-PUFA in muscle

The conversion of the dietary 18:2 n-6 and 18:3 n-3 that escaped from RBH, on their longer-chain derivatives, involves a complex enzymatic system, constituted by ∆5- and ∆6-desaturase enzymes and elongases that performs a series of chain desaturation and elongation reactions (Figure 1.12) (Raes *et al.*, 2004). Enzymes involved in this mechanism are common for both n-3 and n-6 FA, but act preferentially on those of the n-3 series (Brenner, 1989). The first step on conversion of 18:2 n-6 and of 18:3 n-3 is their desaturation by the activity of ∆6-desaturase, which inserts a double bond at the Δ6 position of 18:2 n-6 and of 18:3 n-3. Then it follows a chain elongation and subsequent desaturation by ∆5-desaturase, inserting a double bond at the Δ5 position that generates 20:4 n-6 and 20:5 n-3, respectively. The conversion of 20:5 n-3 to 22:6 n-3 occurs via the Sprecher pathway (Sprecher, Luthria, Mohammed & Baykousheva., 1995) which involves two consecutive elongations, desaturation by ∆6-desaturase and β-oxidation (Alvarenga *et al.*, 2014). Conversion of 20:4 n-6 to longer-chain n-6 follows the same pathways, resulting in 22:5 n-6 as the longer derivative. Retro-conversion of 22:6 n-3 into 22:5 n-3 and 20:5 n-3 is also reported but is considered as a process with low efficiency (Russo, 2009; Calder, 2012). In ruminants and in humans, the conversion of 18:3 n-3 into the correspondent LC-PUFA, is a mechanism with very low efficiency (Scollan *et al.*, 2001; Cooper *et al.*, 2004; Daley *et al.*, 2010), what emphasizes the importance of incorporating in the diets of ruminants not only lipid sources rich in 18:3 n-3, but also of preformed sources of EPA and DHA, as fish oils, in order to increase the deposition of the n−3 LC-PUFA in the meat (Cooper *et al.*, 2004; Bessa *et al.*, 2015).

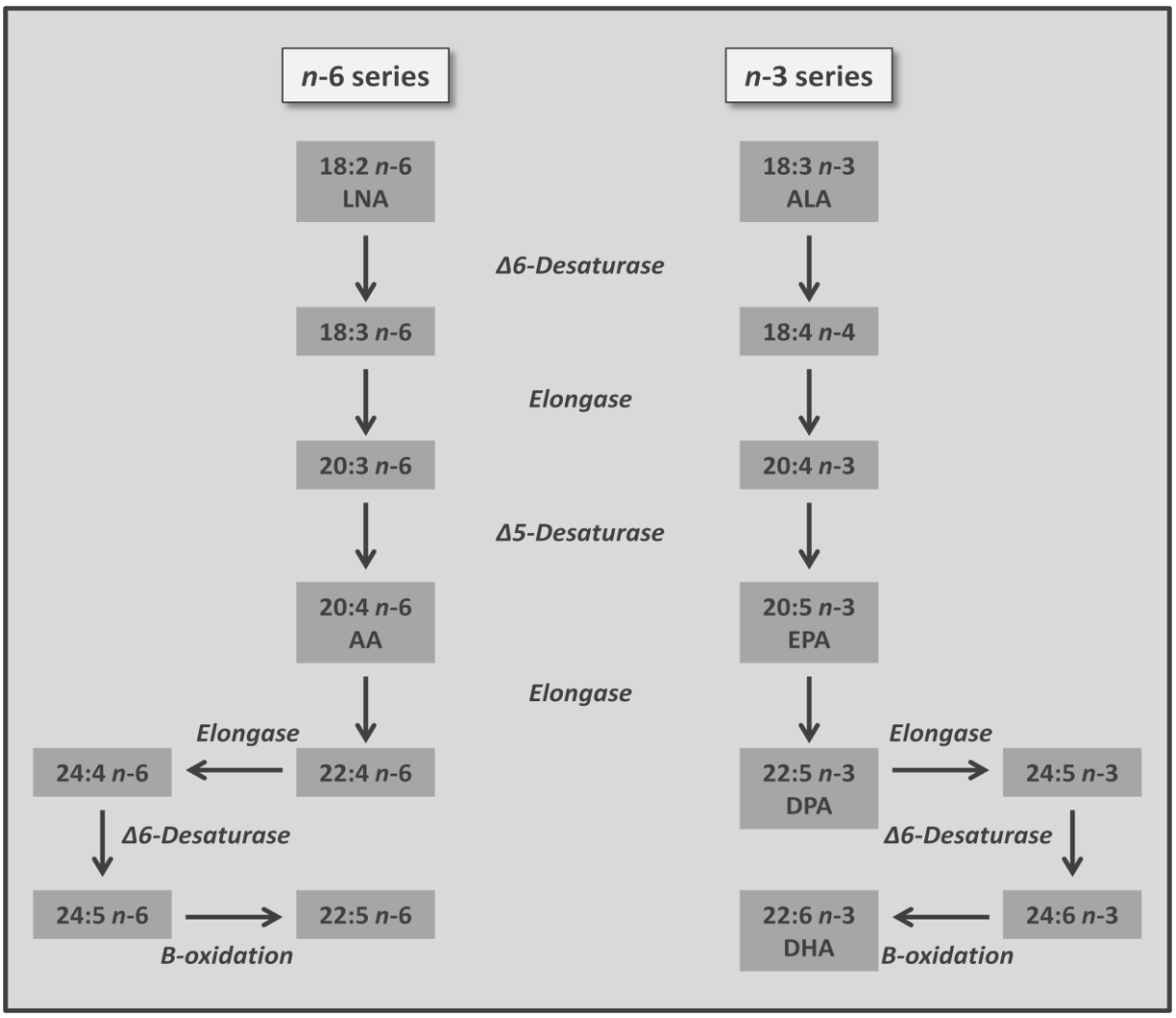


Figure 1.12- General overview of n-6 and n-3 PUFA biosynthesis pathways. Adapted from Barceló-Coblijin and Murphy (2009).

Fatty acid composition of phospholipids is less influenced by dietary factors, although differences in the levels of n-6 and n-3 LC-PUFA are reported (Raes *et al.*, 2004; Scollan *et al.*, 2006). In the first place, the conversion steps of 18:2 n-6 and 18:3 n-3 to the correspondent n-6 and n-3 longer chain PUFA depends of the dietary ratio of n-6 and n-3, due to the competition for desaturases and elongases by the two families of FA (Alvarenga *et al.*, 2014). The regulation of the endogenous synthesis of LC-PUFA by a mechanism of feedback inhibition by the end products is also reported (Calder, 2012). In fact, lipid supplementation of diets with oils rich in n-3 and n-6 PUFA may reduce mRNA expression of the genes that codify for Δ5- and Δ6-desaturases (*FADS1* and *FADS2*, respectively) (Nakamura & Nara, 2002), as well as the activity of Δ5- and Δ6-desaturases as reported in growing cattle (Herdmann *et al.*, 2010). Also TFA as 10*t*,12*c*-18:2 interfere in LC-PUFA synthesis and incorporation into cellular membrane phospholipids, inhibiting Δ5- and Δ6-desaturase activity (Mahfouz, 1981; Kinsella, Bruckner, Mai & Shimp, 1981; Dhaka *et al.*, 2011). Effects of genetic variation between breeds on the content of n-3 and n-6 PUFA in muscle have been identified in cattle and sheep (Sinclair *et al.*, 2007; Alvarenga *et al.*, 2014)

and Hopkins, Fogarty and Mortimer (2011), reviewing the influence of sheep genotype on meat quality traits, reported a moderate heritability (h^2) for the content of EPA and DHA in meat. Also Mortimer *et al.* (2014) estimated moderate values of h^2 (0.17 and 0.22 for EPA and DHA, respectively) for lamb meat. In both studies the authors concluded that the enhancement of the level of n-3 PUFA in meat can be achieved with the development of both genetic and nutritional approaches, but the latter can be more efficient, rapid and easily to perform.

1. 4. Effects of Intramuscular fat on meat quality

Intramuscular fat, also named as marbling fat, is often associated to meat quality perception by consumers'. For consumers, meat must conjugate a high nutritional quality with a high eating satisfaction. In several countries, as in Portugal and the countries of North Europe, high levels of marbling fat is considered as a negative component of meat, leading to a depreciation of its value in the moment of purchase. However, IMF and its FA composition plays an important role in meat organoleptic quality and a minimum of IMF content is needed to guarantee a good eating quality. For lamb meat, there are no specific studies about the preferences of the Portuguese consumers. However, in Australia, Hopkins *et al.* (2011) referred as ideal value of IMF, 5%, which coincides with the reference value for a meat to be considered as a low fat food (Food Advisory Committee [FAC], 1990).

The amount of IMF in meat may influence tenderness and juiciness after cooking and FA composition determines the oxidative stability of muscle and may affect meat colour during storage and flavour. However, while these relationships are strongly observed in some studies being well demonstrative of an important role of IMF in meat eating quality, there are others where only a small contribution of IMF to meat sensory characteristics was observed (Wood *et al.*, 2008).

1.4.1. Meat quality and acceptability by consumer

In the purchase moment the main aspect of meat quality that influences consumer's decision is appearance which depends of meat colour and the level of fat and fat colour. Meat colour is the attribute with major influence in the decision of consumer to purchase a determined piece of red meat because meat discoloration is considered by consumers as indicative of the loss of freshness (Mancini & Hunt, 2005).

The evaluation of meat appearance by consumers is subjective and depends of individual sensibility, personal experience and preference and it is related to consumer's geographic origin, cultural background and gastronomic traditions (Sañudo *et al.*, 1998b; Font i Furnols

et al., 2011). After cooking, the meat sensory attributes related to its texture (tenderness and juiciness), odour and flavour are the most influent in meat acceptability by consumer. In beef, meat acceptability is mostly affected by tenderness, followed by juiciness, while in lamb, that is a meat typically characterized as tender and juicy, flavour is the attribute with more effect on consumer satisfaction. Many pre-slaugther factors such as genetics, sex, age, live weight at slaughter and feeding system influence the meat composition and organoleptic characteristics of meat (Resconi,Campo, Font i Furnols, Montossi & Sañudo, 2009).

1.4.2. Intramuscular fat and meat tenderness and juiciness

In general, tenderness is considered as the most important component of meat sensory quality due to the unacceptability of tough meat. Tenderness mainly depends of the changes in the myofibrilar protein structure of muscle during the period of time between animal slaughter and meat consumption (Wood *et al.*, 1999). Also the presence of fat in muscle can affect tenderness directly and indirectly. High levels of marbling decrease muscle resistance to shearing due to a dilution of muscle fibers by soft tissues (Wood *et al.*, 1999) and also due to the opening muscle structure by the expansion of IMF adipocites in the perimysal connective tissue, which forces the muscle fibre bundles to apart (Wood *et al.*, 1999).
The amount of IMF favors meat juiciness because it is associated to a higher capacity of meat to retain water during the cooking process (Wood *et al.*, 2008). In other way, as tenderness increases with IMF, a tender meat presents a higher capacity to release juices during chewing, thus, higher juiciness. Moreover, the presence of intramuscular fat stimulates saliva secretion during mastication (Resconi *et al.*, 2009), favoring the sensation of juiciness.

1.4.3. Intramuscular fat and meat oxidative stability. Lipid oxidation, meat discoloration and rancidity during storage

Due to the propensity of unsaturated FA to rapidly oxidate, meat lipid composition is directly linked to the meat oxidative stability. The oxidation of lipids, together with that of meat pigments, influences meat shelf-life through its relationship to meat discolouration and the development of rancidity in raw meat during storage (Mottram, 1998; Faustman, Sun, Mancini & Suman, 2010). During cooking, meat also suffers a thermal induced lipid oxidation, but that rapid lipid degradation is, until a certain level, desirable, as it plays a crucial role on the development of the characteristic meat flavour (Mottram, 1998; Wood *et al.*, 2003; Resconi, Escudero & Campo, 2013). Thus, the stability of meat FA is an important factor that

influences meat quality as during the purchase (colour) as in the consumption (flavour) with impact on consumer appeal and satisfaction.

A high proportion of PUFA in muscle increases meat susceptibility to oxidation (Morrissey, Sheehy, Galvin, Kerry & Buckley, 1998). The first step of lipid oxidation depends of the remotion of hydrogen from a methylene carbon in the FA and the presence of carbon double bonds in PUFAs weaks the carbon-hydrogen bonds, making that hydrogen to be easily removed (Nieto & Ros, 2012). As PUFA are preferentially deposited in the phospholipid fraction of muscle, lean meat, with low level of IMF and thus a higher phospholipid proportion, is more susceptible to oxidation and thus less stable than meat with a higher fat content. Among PUFA, the n-3 PUFA, especially n-3 LC-PUFA are reported as to be the less stable (Morrissey *et al.*, 1998; Nute *et al.*, 2007; Wood *et al.*, 2008). Wood *et al.* (2003) concluded that lipid and colour stability of beef is only affected by linolenic acid concentration if it approaches from 3% of total lipids muscle. Lipid oxidative stability not only depends from the level of n-3 PUFA *per se*, but also from the total of n-6 PUFA, the concentration of antioxidants, such as vitamin E, the availability of the heme iron in muscle and the ratios between these factors (Buckley, Morrissey & Gray, 1995; Moloney, Kennedy, Noci, Monahan & Kerry, 2012; Ponnampalam, Butler, McDonagh, Jacobs & Hopkins, 2012; Ponnampalam *et al.*, 2014). A positive effect of CLA in the reduction of oxidation, improving meat shelf-life, due to its antioxidant properties, has been proposed by several authors (Du, Ahn, Nam & Sell, 2000; Joo, Lee, Ha, & Park, 2002; Mir *et al.*, 2002; Hur *et al.*, 2004). However, the correlation between CLA and the reduction of meat lipid oxidation is not always clear as it was reported by Moloney *et al.* (2012).

Lipid peroxidation can originate hydroperoxides and conjugated dienes and secondarily, isoprostanes, prostaglandin (PG) F2-like compounds, carbonyls (ketones and aldehydes), alcohols, hydrocarbons, isofurans, epoxides as malondialdehyde (Min & Ahn, 2005; Faustman *et al.*, 2010). Some of these products (as unsaturated aldehydes) promote the myoglobin oxidation, resulting in meat discoloration (Faustman *et al.*, 2010), while others (volatile organic compounds (VOC)) are linked to the formation of rancidity in raw meat and with the development of the meat flavour during cooking (Mottram, 1998; Resconi *et al.*, 2013).

Lipid oxidative stability of foods can be assessed through the thiobarbituric acid reacting substances (TBARS) test which measures the malondialdehyde (MDA) produced by lipid oxidation (Wood *et al.*, 2008). For ruminants, Campo *et al.* (2006) concluded that the threshold for detection of the negative effects of lipid oxidation on beef quality was of 2.28 mgMDA/kg while Soldatou, Nerantzaki, Nontominas and Savvaidis (2009) concluded that lamb meat rancification could only be detected for TBARS levels higher than 4.4 mgMDA/kg.

Fresh and appellative meat must have a high redness (a*) indice value, to be bright in colour and present higher colour saturation (Chroma) (Mancini & Hunt, 2005). However, the redness appreciation, independently of the direct association of red to fresh meat, also depends of the species and slaughter weight (*eg.*, beef *vs* veal, mutton *vs* light lamb) and of the consumer's profile. In the European Mediterranean countries, the preference for lamb meat color is pale pink, because it is associated by consumers to youthfulness. Lamb meat with high redness scores is associated to old lambs or even to adult animals and is depreciated by consumers (Juarez *et al.*, 2009). Even, inside a country there are differences in consumer preferences at regional level, depending on geographic specificities which are associated to the traditional production system and to the type of lamb that is produced and the consumer´s gastronomic pratices (Montossi *et al.*, 2013).

Meat colour depends of the myoglobin concentration and of the proportions of the different chemical myoglobin forms present in muscle. Deoxygenated myoglobin (DeoxyMb) is the form of myoglobin in anaerobic conditions and is purple, oxymyoglobin (OxyMb) is the oxygenate form of myoglobin and it presents a bright cherry red colour and the oxidation of myoglobin forms the metamyoglobin (MetMb), which is brown (Mancini & Hunt, 2005).

Meat discoloration occurs in the surface of meat exposed to oxygen and involves the convertion of the OxyMb to MetMb, due to a decrease in heme redox stability and consequent oxidation of OxyMb. This oxidative process leads to a decrease in meat redness (a*) followed by an increase of meat browning, expressed by increasing values of Hue angle (H*) over time of storage (Mancini & Hunt, 2005; Morrissey, Jacob & Pluske, 2008; Faustman *et al.*, 2010; Calnan, Jacob, Pethick & Gardner, 2014). Meat is considered stable in colour if remains red during display. Lamb meat is less stable than beef what can be related to differences on the primary structure of mioglobin, which interact with aldeyde compounds produced in oxidation (Faustman *et al.*, 2010).

Meat discoloration and lipid oxidation processes influences each other. Specific products of lipid oxidation such as α,β unsaturated aldeydes and 4-hydroxynonenal (HNE) are directly related to the increase of OxyMb oxidation (Faustman *et al.*, 2010). In turn, the oxidation of OxyMb to MetMb, form reactive intermediary products such as superoxide anion and iron, that contribute to enhancing lipid oxidation (Baron & Andersen, 2002; Faustman *et al.*, 2010). However, the relationship between lipid and mioglobin oxidation processes it is not already fully understood and a lack of a clear relation between those oxidative processes in meat has been reported in several studies (Luciano *et al.*, 2009a; Faustman *et al.*, 2010; Jerónimo *et al.*, 2012; Kasapidou *et al.*, 2012; Moloney *et al.*, 2012; Ponnampalam *et al.*, 2012). Oxidation of muscle lipids and myoglobin is a complex mechanism and depends on the balance between diverse factors as the concentration of antioxidant compounds in muscle,

such as vitamin E, and the level and proportions of different PUFA on IMF (*e.g.*, 18:3n-3 vs 18:2 n-6; n-3 PUFA *vs* n-6 PUFA) and myoglobin and heme and non heme iron concentration in muscle. Incongruent results may also be due to a higher resistance of myoglobin/colour to oxidation than lipids (Moloney *et al.*, 2012; Kasapidou *et al.*, 2012).

In parallel with meat discoloration, lipid oxidation also leads to the rancidity of raw meat during storage, which will affect the acceptability of meat flavour and odour after cooking (Wood *et al.*, 2003, Resconi *et al.*, 2013). The rancid flavour and odour of meat, that is sometimes referred to as warmed-over flavour, is apparently related to the dominance of alkanal (hexanal, nonanal) or some alcohols (1-penten-3-ol, 1-octen-3-ol) (Young, Berdagué, Vialloq, Rousset-Akrim & Theriez, 1997; Calkins & Hodgen, 2007; Nieto & Ros, 2012) originated by lipid oxidation during storage, being hexanal and 1-octen-3-ol specific products from the 18:2 n-6 oxidation (Min & Ahn, 2005).

1.4.4. Intramuscular fat and meat flavour and odour during cooking

Meat flavour has been considered as the third sensory attribute, after tenderness and juiciness, which affects meat palatability and consumption (Calkins & Hodgen, 2007). However, when tenderness is constant, flavour is reported as the most important factor that influence consumer preferences and purchase decision (Killinger, Calkins, Umberger, Feuz & Eskridge, 2004; Sitz, Calkins, Feuz, Umberger & Eskridge, 2005). This is the case of lamb meat, where flavour is often assumed by consumers as the main factor that influences their satisfaction during consumption of this meat (Young *et al.*, 1997, Duckett & Kuber, 2001). Moreover, small changes in sensory ratings for flavour, have a high influence in meat overall acceptability (Platter *et al.*, 2003).

Flavour is a meat attribute highly complex and its development and influence in meat palatability and acceptability has been the subject of numerous studies along the years. Examples of specific published revisions on this subject are: Farmer (1994), Mottram (1998), Priolo, Micol and Agabriel (2001), Channon, Lyons and Bruce (2003), Vasta and Priolo (2006), Calkins and Hodgen (2007), Dransfield (2008), Resconi *et al.* (2013) and Watkins, Frank, Singh, Young and Warner (2013).

Raw meat aroma is little intense, presenting a blood-like taste (Mottram, 1998). Meat flavour is produced during the cooking process by a complex series of thermally induced reactions involving the non-volatile components of muscle and fat, such as Maillard reactions, between amino acids and reducing sugars, and the thermal induced lipid degradation, respectively (Mottram, 1998). The water-soluble components of the lean tissue are precursors of volatile compounds such as furans, pyrazines, pyrroles, oxazoles, thiophenes, thiazoles and others (Mottram, 1998). These volatiles are related to the meaty flavour, which is transversal to all

cooked meats. The volatile compounds derived from the lipid oxidation during heating (lipid-derived volatiles) are largely associated to the species-specific flavour and odour (Farmer *et al.*, 1994; Mottram, 1998; Watkins *et al.*, 2013)) and include aldehydes and ketones as the main compound groups. However, aliphatic hydrocarbons, alcohols, lactones, carboxylic acids and esters are also important components (Young *et al.*, 1997; Mottram, 1998; Elmore, Mottram, Enser & Wood, 2000). Some of the lipid-derived volatiles and those generated by the Maillard reactions, may interact with each other and also with the volatile products resultant of meat oxidation process during storage, being their products an additional source of intermediates for further flavour-forming reactions (Resconi *et al.*, 2013).

The influence of IMF content and FA profile in the development of volatiles and their effect on meat flavour has been investigated by several authors (*e.g.* Elmore, Mottram, Enser & Wood, 1999; Elmore *et al*, 2000; Campo *et al.*, 2003; Young, Lane, Priolo & Fraser, 2003; Elmore *et al.*, 2005; Nute *et al.*, 2007; Resconi *et al.*, 2010; Van Ba, Amna & Hwang, 2013). The type and content of fat derived volatiles produced during cooking depends on the IMF fatty acid profile and thus, also the flavours and odours found in meat. Due to their high chemical instability and susceptibility to oxidation (Morrissey *et al.*, 1998), PUFA are particularly important in meat flavour development. Therefore a meat containing highly unsaturated IMF will be more susceptible to the development of volatiles and flavours associated to UFA oxidation. The 18:3 n-3 degradation produces aldehydes such as 4-heptenal, 2,4-heptadienal and 2,6-nonadienal, 2-hexenal, propanal, 2,4,7-decatrienal, alcohols as 1,5-octadien-3-ol, 2,5-octadien-1-ol, and ketones as 1,5-octadien-3-one (Young *et al.*, 1997; Min & Ahn, 2005; Calkins & Hodgen, 2007). Eicosapentaenoic acid (20:5 n-3) and docasahexaenoic acid (22:6 n-3) are related to the synthesis of highly unsaturated volatiles such as octatriene, 2-ethylfuran, 2-(2-pentenyl)-furan, 1-penten-3-ol and 2-ethylpyridine (Elmore *et al.*, 2000). Grassy, nuts, fishy, linseed/putty and creosote are some of the offfavours associated to 18:3 n-3 and derivative FA (Campo *et al.*, 2003; Calkins & Hodgen, 2007). In turn, HNE, hexanal, 2-heptenal, 2,4-decadienal, 2-nonenal, octanal and 1-octen-3-ol are reported as originated by 18:2 n-6 (Young *et al.*, 1997, 2003; Raes *et al.*, 2003; Min & Ahn, 2005) and are associated to a wide range of offflavours, including fatty, grassy, fishy, fried, soapy, lemon, almond, cardboardy, paper or mushrooms, among others (Calkins & Hodgen, 2007; Watkins *et al*., 2013). Blank, Lin, Vera, Welti and Fay (2001) reported that *trans*-4,5-epoxy-(E)-2-decenal, 1-octen-3-one and 2,4-decadienal are the three main aroma compounds from 20:4 n-6 oxidation. Nevertheless, the effects of volatiles on meat flavour are directly linked to their concentration. For example, hexanal and 2,4-decadienal usually contribute positively to beef flavour, although, at higher concentrations are associated to off flavours as grassy, fatty or tallow (hexanal) and fatty, fried (2,4-decadienal) (Calkins & Hodgen 2007, Watkins *et al.*, 2013).

Branched chain FA (BCFAs), especially those of medium chain length (8-10 carbon atoms), have also an important role in the development of sheep meat odour and flavour (Wong, Nixon & Johnson, 1975; Young *et al.*, 1997). These FA result from the metabolization of propionate originated by the ruminal fermentation of dietary carbohydrates (Young *et al.*, 1997, Berthelot, Pierzynowski, Sauvant & Kristensen, 2002) and have been associated to the development of the odour and flavour characteristic of sheep meat (muttony) and goat (goaty) (Wong *et al.*, 1975; Brennand & Lindsay, 1992; Young *et al.*, 1997). The BCFA with more relevance for the sheep meat and goat meat specific flavour and odour are 4-methyloctanoic, 4-methylnonanoic, 4-ethyloctanoic and 4-ethylnonanoic acids (Wong *et al.*, 1975; Brennand & Lindsay, 1992; Young *et al.*, 1997). However, as BCFA are preferentially deposited in TAG (Brennand & Lindsay, 1992), the direct effect of these FA in meat flavour will have more impact in the meat of lambs with high levels of IMF.

The influence of fat in meat flavour, its perception and acceptability by consumer, depends of a combination of several aspects related to meat itself (texture, fat content), the cooking method and heating conditions (*eg.,* oven vs grill, temperature, duration of heating) and the consumer (olfactory, gustatory and nociceptive capacities, consumption habits and preferences associated to geographic, ethnic and cultural differences) (Sañudo *et al*.,1998a, 1998b, 2000; Calkins & Hodgen, 2007; Dransfield, 2008, Watkins *et al*., 2013). Thus, understanding the mechanisms by which intramuscular fat affects flavour perception and acceptability of cooked meat it is gaining more importance in meat research, in order to conciliate health benefits to a pleasant eating experience, fulfilling consumers´ expectative.

1.5. Nutritional strategies to modulate ruminant lipid metabolism in order to improve meat nutritional quality

In the last few years, the demand for healthier and functional foods has been significantly increased by the consumers. A “functional” food contains a “component (whether a nutrient or not) which affects one or more targeted functions in the body in a positive way” (International Life Sciences Institute [ILSI]), 1999). The enrichment of ruminant’s meat in bioactive FA with healthy (or expected healthy) functions such as 11*t*-18:1, 9*c*,11*t*-18:2 and n-3 PUFA, especially n-3 LC-PUFA, improves the nutritional value of meat fat and may justify the recognition of that meat as a functional food. Thus, several dietary strategies involving the manipulation of the RBH of dietary PUFA and the promotion of absorption and deposition of the target FA in IMF are being used to improve the nutritional value and, simultaneously, the functional properties of meat from ruminants. Moreover, consumers are paying further attention to the sensory attributes of meat, such as the meat colour in the

purchase moment and meat tenderness, juiciness and flavour after cooking. In consequence, meat industry is preferentially requiring a final product which conjugates a high nutritional value with high stability during storage time and higher patterns of palatability to correspond to consumer´s expectations.

1.5.2. Diet composition

High-forage diets vs concentrate-based diets

Diets based on pasture, hay or grass silage, are generally associated to the production of a meat with an IMF content of typically 2-5%, and a healthier lipid profile which is characterized by a high proportion of 11*t*-18:1 and moderate proportions of 9*c*,11*t*-18:2 and of n-3 PUFA, including n-3 LC-PUFA (Daley *et al.*, 2010; Scollan *et al.*, 2006; 2014; Howes, Bekhit, Burritt & Campbell, 2015). The higher activity of rumen cellulolytic microorganisms and the ruminal conditions established during fiber fermentation leads to an extensive RBH resulting in a high production and accumulation of 11*t*-18:1. Thus, feeding growing ruminants with diets rich in forage allows the increase of 11*t*-18:1 availability to its endogenous conversion in 9*c*,11*t*-18:2 (Bessa *et al.*, 2015). In such conditions, however, the endogenous synthesis of 9*c*,11*t*-18:2 in intramuscular fat may probably be limited by the *SCD* mRNA expression which, in this type of diets, with low starch content and thus low insulin levels, is down-regulated (Figure 1.13) (Daniel *et al.*, 2004; Sinclair, 2007; Bessa *et al.*, 2015). Other constraint to the availability of 9*c*,11*t*-18:2 in meat from ruminants fed with high levels of forage, is the low energy content of these diets. Rumenic acid is preferentially deposited in TAG fraction of intramuscular fat (Jerónimo *et al.*, 2011). When intramuscular fat deposition, and consequently TAG fraction, are reduced, also the deposition of 9*c*,11*t*-18:2 is compromised (Figure 1.13) (Bessa *et al.*, 2015).

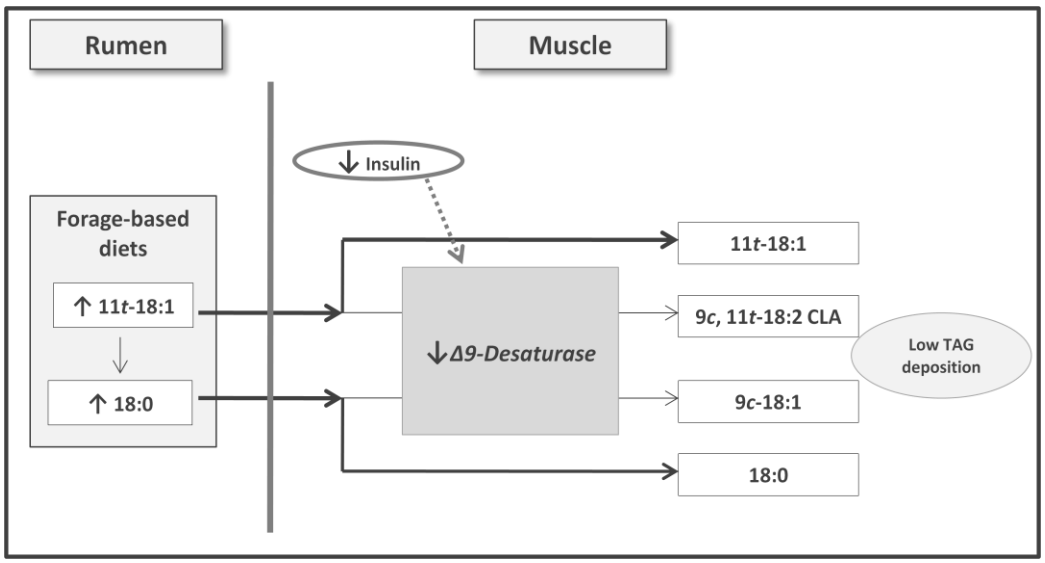


Figure 1.13 – Main limitations to 9*c*,11*t*-18:2 deposition in meat from ruminants fed forage-based diets. TAG: Triacylglicerols. Adapted from Bessa *et al.* (2015).

Grasses and forages are the most important sources of 18:3 n-3 for ruminants (Raes *et al.*, 2004; Hess, Moss & Rule, 2008) and despite of RBH effects, it is well established that feeding ruminants with forage-rich diets, especially with fresh grass, results in a higher content of 18:3 n-3 and its longer chain derivatives, mainly EPA, in meat (Fisher *et al.*, 2000; Santos-Silva *et al.*, 2002a, Bessa *et al.*, 2005, Bessa *et al.*, 2008; Alfaia *et al.*, 2009). Lean meat from grazing ruminants is also often associated to a higher content on PUFA, comparatively to SFA (Scollan *et al.*, 2006; Daley *et al.*, 2010; Howes *et al.*, 2015). Since the phospholipid content is relatively constant in muscle and independent of total fat content, meat with low IMF have a high proportion of PL and consequently of PUFA, which are mainly associated to phospholipids (Raes *et al.*, 2004; Wood *et al.*, 2008).

Due to the low energy content of grass-based diets, growing animals often present lower feed efficiency than those fed concentrates, which negatively affects their growth performance, carcass fatness and meat sensorial attributes such as tenderness and juiciness (Sañudo *et al.*, 1998b; Priolo, Micol, Agabriel, Prache & Dransfield, 2002a; Daley *et al.*, 2010). Meat from ruminants fed with high-forage diets usually presents a higher intensity to species-specific odour and flavour than meat from ruminants fed with concentrate-based diets (Sañudo *et al.*, 2000; Duckett & Kuber, 2001; Priolo *et al.*, 2001; Channon *et al.*, 2003; Young *et al.*, 2003; Resconi *et al.*, 2010; Watkins *et al.*, 2013). Offflavours associated to the meat lipid composition and oxidation such as “greasy”, “fishy” and “rancid” and/or to the plant composition as “pastoral”/ “grassy”/“green” are frequently reported by trained sensory panels in the meat from pasture animals (Priolo *et al.*, 2001, 2002a; Young *et al.*, 2003; Scollan *et al.*, 2006; Calkins & Hodgen, 2007; Sinclair *et al.*, 2007; Watkins *et al.*, 2013). The high susceptibility of n-3 PUFA to oxidize could also potentially reduce meat oxidative stability

during storage. Nevertheless, the high content of meat in antioxidants such as vitamin E, which is naturally present on grass, inhibits, or at least, attenuates, the negative effects attributed to n-3 PUFA oxidation on meat color and lipid stability on meat from ruminants fed with forages (Scollan *et al.*, 2006; Wood *et al.*, 2008; Daley *et al.*, 2010; Luciano *et al.*, 2011a; Scollan *et al.*, 2014).

Meat lipid composition of growing ruminants fed with high forage diets can be quite variable, depending on several factors: proportion of grass/forage in the diet; botanical composition; plant growth stage; feeding on pasture or with preserved forages; and the preservation method (hay or silage) (Scollan *et al.*, 2006; Sinclair, 2007; Wood *et al.*, 2008; Howes *et al.*, 2015). Time on grass/forage-based diet is also important because it affects fat deposition and the changes in the FA composition of muscle and adipose tissue (Scollan *et al.*, 2006; Sinclair, 2007; Bessa *et al.*, 2008; Howes *et al.*, 2015; Shingfield *et al.*, 2013). The daily grazing period may also influence meat FA composition as it was reported with grazing lambs by Vasta *et al.* (2012), which concluded that animals kept at pasture during 4 h in the afternoon (from 1 pm to 5 pm) produced a meat with a healthier fatty acid profile, including a higher proportion of 9*c*,11*t*-18:2, than that of lambs that grazed 8h during the day (from 9 am to 5 pm) and 4h in the morning (from 9 am to 1 pm) .

The introduction of concentrate feeds in the diet of growing ruminants has increased in many parts of the World (Montossi *et al.*, 2013). Concentrates have a high metabolizable energy density, mainly due to the starch from the cereal grains, and they can be used by farmers under several conditions. In the pasture-based systems, concentrate feeds are mostly restricted to the supplementation of animals during the seasons with low grass availability. However, with the intensification of the meat producing system and in order to improve feed efficiency, growth performances, carcass fatness and meat sensory attributes, the proportion of concentrates in ruminant diets may be quite high, close to be the exclusive feed (Montossi *et al.*, 2013).

However, the use of concentrates for growing ruminants, affects meat fatty acid composition. A low content on 11*t*-18:1 and 9*c*,11*t*-18:2 and high levels of 9*c*-18:1, 10*t*-18:1 and n-6 PUFA characterize the meat lipid profile from high-concentrated fed ruminants (Wood *et al.*, 2008; Daley *et al.*, 2010). In spite of the high insulin levels and the high TAG deposition, the changes in rumen microbial populations of cellulolytic by amilolytic microorganisms cause a change in the normal pattern of RBH, frequently called as "*trans*-10 shift". In such conditions is favored the synthesis and accumulation on rumen of 10*t*-18:1 and is reduced the availability of 11*t*-18:1, limiting the conversion, and consequently the concentration, of 9*c*,11*t*-18:2 in IMF. Therefore, occurs a higher deposition of 10*t*-18:1 instead that of 11*t*-18:1 in IMF and, due to the increased activity of ∆9-desaturase, a high deposition of 9*c*-18:1 (Figure

1.14) (Bessa *et al.*, 2005; Rosa *et al.*, 2014; Bessa *et al.*, 2015). In the other hand, as concentrate feeds are rich in 18:2 n-6 the incorporation on muscle of n-6 FA and its long chain products is high (Wood *et al.*, 2008; Shingfield *et al.*, 2013).

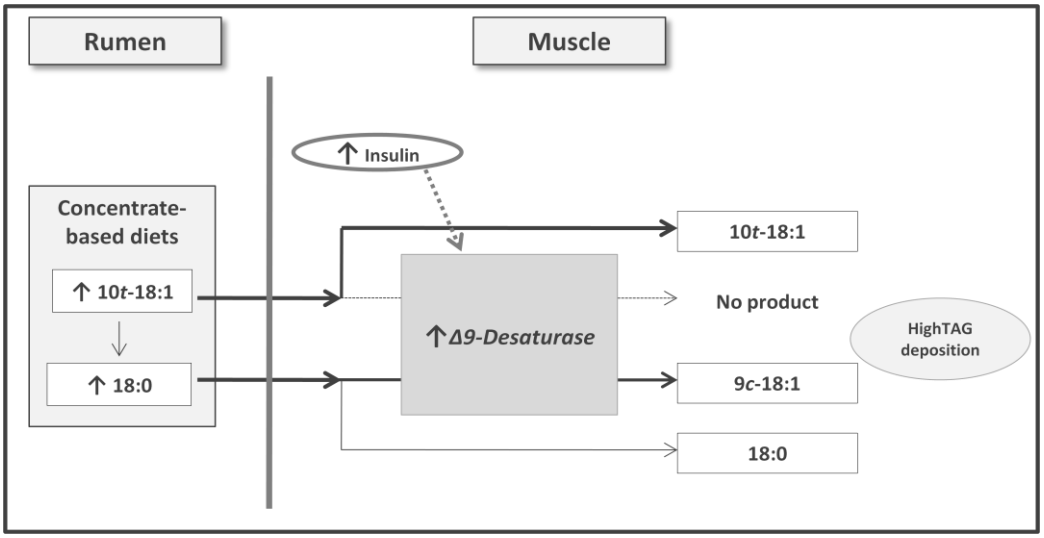


Figure 1.14 – Main limitations to 9*c*,11*t*-18:2 deposition in meat from ruminants fed concentrate-based diets. TAG: Triacylglicerols. Adapted from Bessa *et al.* (2015).

Nevertheless, the nutritional value of meat fat from ruminants fed with concentrate-based diets may be improved through dietary manipulation. To potentiate its content on 9*c*,11*t*-18:2 and on 11*t*-18:1, different nutritional strategies involving a) the increase of substrates availability to RBH, b) the decrease of *trans*-10 shift through the modulation of rumen environment and RBH pathways and c) the increase of ∆9-desaturase activity on muscle, are being under intensive research as reported on several recent reviews (Shingfield *et al.*, 2013; Scollan *et al.*, 2014; Mapiye *et al.*, 2015). Furthermore, the supplementation of high-grain diets with n-3 PUFA sources, such as of 18:3 n-3, and of EPA and DHA it also allows that a higher proportion of those FA escape from RBH and be deposited in IMF, increasing n-3 PUFA content in meat from feedlot ruminants (Scollan *et al.*, 2006; Sinclair, 2007; Shingfield *et al.*, 2013; Scollan *et al.*, 2014; Alvarenga *et al.*, 2015).

Replacing starch in diet with non-starch dietary sources

High starch content in the diets is directly associated to low ruminal pH values which in turn are related to the ruminal conditions that favours the RBH pathways associated to 10*t*-18:1 (Bessa *et al.*, 2015). Thus, the replacement of starch as main energy source of the concentrate-based diets with non-starchy dietary sources can potentially be a usefull strategy

to reduce the occurrence of *trans*-10 shift in the rumen and possibly increasing the 9*c*,11*t*-18:2 and 11*t*-18:1 delivery from RBH. Alternative feed sources rich in energetic carbohydrates others than starch, that can be used include agro-industrial by-products from the juices and sugar industry, such as citrus pulp (CP) and sugar beet pulp, respectively, and soybean hulls which is a by-product of the soybean processing industry. Moreover, these feed resources have been successfully introduced in ruminant diets (Vasta, Nudda, Cannas, Lanza & Priolo, 2008; Bampidis & Robinson, 2006). In the present study it was only approached the use of citrus pulp as alternative feed source to cereals in the diet of lambs.

Dehydrated citrus pulp as alternative energy source to starch on concentrate-based diets as a strategy to* avoid *trans*-10 *shift

Citrus pulp is an agro-industrial by-product of the juice industry which is widely available for the industry of compound feeds for ruminants as dehydrated citrus pulp pellets (DCP). Dehydrated citrus pulp is formed by shedding, liming, pressing and drying the peel, pulp, seed residues and culled fruits to about 80 g/kg moisture (Bampidis & Robinson, 2006). Although the nutritional composition of CP depends of several factors such as the species and variety of the fruit, its maturity degree, the climatic conditions of production and the processing methods (Bampidis & Robinson, 2006), this by-product is characterized by a low protein and starch content being rich in readily and highly fermentable carbohydrates such as pectin and sugars (Bampidis & Robinson, 2006).

Pectin fermentation produces less lactate than that of starch and thus, DCP presents a fermentative profile similar to forages (Ariza, Bach, Stern & Hall, 2001). Therefore, the replacement of starch by pectin as the main source of energy for rumen microbes leads to a rapid microbial growth (Bampidis & Robinson, 2006), and simultaneously prevents the rapid descent of rumen pH, favouring the activity of cellulolytic bacteria and fibre degradability (Ben-Ghedalia, Yosef, Miron & Est, 1989). As result, is established a rumen environment which favours the biohydrogenation pathways for the synthesis of 11*t*-18:1 and 9*c*,11*t*-18:2 (Zened, Enjalbert, Nicot & Troegeler-Meynadier, 2013). Otherwise, the antimicrobial capacity of some of the bioactive components present in CP, such as phenolic compounds, and the 18:3 n-3 content which is higher in DCP than in cereals (Rodrigues *et al.*, 2010), may also result in an improvement of meat lipid profile as it was suggested by Lanza *et al.* (2015).

It has been reported that DCP can replace grain or forage in small ruminant diets without comprome animal performance (Bhattacharya & Harb, 1973; Rodrigues *et al.*, 2008a; Bueno, Santos, Cunha, Neto & Verissímo, 2004) or meat quality (Lanza, Priolo, Biondi, Bella & Ben Salem, 2001; Scerra, Caparra, Foti, Lanza & Priolo, 2001; Caparra, Foti, Scerra, Sinatra & Scerra, 2007; Rodrigues *et al.*, 2008b). Furthermore, due to its high levels of bioactive

compounds with antioxidant action, which includes phenolics, flavonoids, carotenoids and ascorbic acid (Abeysinghe *et al.*, 2007), dietary DCP may increase meat oxidative stability as it was reported by Simitzis, Ilias-Dimopoulos, Charismiadou, Biniari and Deligeorgis (2013) and by Inserra *et al.* (2014). This fact represents other advantage from the use of this feed source in the production of meat from ruminants reared in intensive system, which generally, has a high susceptibility to oxidative deterioration, due to the low content of concentrates in antioxidant substances (Luciano *et al.*, 2012).

1.5.3. Dietary lipid supplementation with polyunsaturated fatty acids

There are two main purposes for supplementing ruminant diets with fat: incrementing the energy density of the diet, due to the high caloric contentof fats (9 kcal/g) and manipulation of FA composition of products. Concerning this last aspect, dietary PUFA supplementation increases the concentration of subtracts to RBH and also the C18 BI formation namely 11*t*-18:1 and CLAs as 9*c*,11*t*-18:2. In other way, as not all dietary PUFA are converted in their saturated forms, the proportion of dietary PUFA and BI escaping the rumen increases, becoming available to be deposited in tissues. Therefore, the addition of oils and feed sources rich in PUFA to ruminant diets is an effective approach to improve the nutritional value of meat through the manipulation of rumen lipid metabolism, allowing the reduction of SFA:PUFA ratio and the promotion of meat enrichment in healthy *trans* FA (11*t*-18:1 and 9*c*,11*t*-18:2) and in n-3 PUFA (Sinclair, 2007). However, it must be noted that the effects on 9*c*,11*t*-18:2 and 11*t*-18:1 are highly dependent of the forage:concentrate ratio of the diet, due to *trans*-10 shift. Vegetable oils (*e.g.* linseed oil, sunflower oil, soybean oil), oilseeds, (*e.g.* rapeseed, linseed, soybean and sunflower seed), marine fish oil and marine algae are the main lipid sources that can be used in the supplementation of ruminant diets (Scollan *et al.*, 2006) and their effects in the improvement of meat and milk FA composition and the development of technological methods to protect dietary PUFA from RBH is under intensive research (Shingfield *et al.*, 2013; Scollan *et al.*, 2014; Mapiye *et al.*, 2015).

Supplementing forage diets with 18:2 n-6 rich sources (*e.g.* soybean oil, sunflower seed and sunflower oil, safflower seeds), depending of the non-occurrence of *trans*-10 shift, is an efficient strategy to increase 9*c*,11*t*-18:2 content in muscle (Bolte *et al.*, 2002; Wachira *et al.*, 2002; Santos-Silva *et al.*, 2003, 2004; Bessa *et al.*, 2005; Boles *et al.*, 2005; Jerónimo, Alves, Prates, Santos-Silva & Bessa, 2009). The supplementation with 18:2 n-6 generally presents a neutral effect on the SFA in muscle while 18:2 n-6 increases, resulting in an improvement on PUFA:SFA ratio (Sinclair, 2007). Fat supplements rich in n-3 PUFA, such as linseed and linseed oil mainly improves meat concentration of 18:3 n-3 and also of n-3 LC-PUFA (Scollan *et al.*, 2001; Wachira *et al.*, 2002; Cooper *et al.*, 2004; Demirel *et al.*, 2004; Bessa *et al.*,

2007; Jerónimo *et al.*, 2009). However, as the efficiency of conversion of 18:3 n-3 into EPA and DHA is very low, (Sinclair, 2007), the supplementation of ruminant diets with pre-formed sources of EPA and DHA, such as fish oil and marine algae, is the most effective way to increase in n-3 LC-PUFA on meat (Scollan *et al.*, 2001; Ponnampalam *et al.*, 2001; Wachira *et al.*, 2002; Cooper *et al.*, 2004; Demirel *et al.*, 2004; Díaz *et al.*, 2011). Dietary supplementation with sources of n-3 PUFA may increase 9*c*,11*t*-18:2 in muscle (Bessa *et al.*, 2007; Noci *et al.*, 2007). However, the efficiency of such strategy is lower comparatively to the supplementation with other lipid sources rich in 18:2 n-6 PUFA (Bessa *et al.*, 2007). The ruminal production of 9*c*,11*t*-18:2 from 18:3 n-3 is lower then from 18:2 n-6 (Lee & Jenkins, 2011) and 18:3 n-3 has a negative effect on Δ9-desaturase (Waters *et al.*, 2009; Corazzin *et al.*, 2013). Nevertheless, a simultaneous enrichment in 9*c*,11*t*-18:2 and n-3 PUFA in meat can be achieved by blending lipid sources with different n-6 and n-3 PUFA content (Bessa *et al.*, 2007; Jerónimo *et al.*, 2009; Jerónimo, 2010a, 2010b; Ferreira *et al.*, 2014). However, the effectiveness of lipid supplementation in improving FA composition of ruminants IMF depends on several factors such as the nature and level of lipid supplementation (Scollan *et al.*, 2001; Bolte *et al.*, 2002; Wachira *et al.*, 2002; Santos-Silva *et al.*, 2003; Cooper *et al.*, 2004; Elmore *et al.*, 2005; Noci *et al.*, 2007; Jerónimo *et al.*, 2009, Ferreira *et al.*, 2014), the composition of the basal diet (Bessa *et al.*, 2005) as well and the duration of the period of lipid supplementation (Bessa *et al.*, 2008).

To prevent the negative effects of the supplementation of ruminant diets with high levels of fat on feed intake, rumen fiber digestibility, fatty acid digestibility and oxidative processes, a maximum limit of 16-20% of metabolizable energy intake is accepted (Palmquist, 1994), what represents, approximately, a fat intake close to 60g/kg of dry matter consumed (Scollan *et al.*, 2006). Lipid supplementation can also increase carcass adiposity in detriment of muscle proportion (Wachira *et al.*, 2002; Santos-Silva *et al.*, 2004; Bessa *et al.*, 2005, 2008; Jerónimo *et al.*, 2010a).

The effects of dietary PUFA supplementation on meat shelf life and eating quality must be taken into account due to the high susceptibility of PUFA, and particularly of n-3 PUFA, to oxidative degradation. Reduced lipid oxidative stability promotes negative changes on meat colour and stimulates the development of volatile compounds associated to off-flavours in the cooked meat. These negative effects were reported in meat from ruminants fed diets with high PUFA levels (Elmore *et al.*, 1999, 2000; Duckett & Kuber, 2001; Elmore *et al.*, 2005; Scollan *et al.*, 2006; Nute *et al.*, 2007). The intensity of off-flavours as “greasy” and “fishy” increase with the level of n-3 PUFA in meat (Scollan *et al.*, 2006; Watkins *et al.*, 2013), and the development of adverse flavours and the reduction of colour stability is particularly significant in meat from ruminants fed with diets supplemented with high levels of EPA and DHA from marine oil sources (Elmore *et al.*, 2000; Wood *et al.*, 2003; Elmore *et al.*, 2005;

Díaz *et al.*, 2011). Thus, despite the improvement of ruminant´s nutritional value of fat promoted by the dietary lipid supplementation, meat shelf life and cooked meat quality and the acceptability by consumers may be compromised (Scollan *et al.*, 2006; Watkins *et al.*, 2013). To prevent these constraints, diet may be reinforced in antioxidant compounds, preferentially those from natural origin, such as vitamins E and C, flavonoids and carotenoids, among others (Gobert, *et al.*, 2010; Jerónimo *et al.*, 2012; Simitzis *et al.*, 2013; Inserra *et al.*, 2014; Scollan *et al.*, 2014). The addition of antioxidants to PUFA-enriched compound feeds is particularly important to protect meat quality (Wood & Enser, 1997; Scollan *et al.*, 2006), due to the low content of antioxidant substances in those diets (Luciano *et al.*, 2012).

1.5.4. Plant secondary compounds as feed additives to modulate the fatty acid profile and oxidative stability of meat - condensed tannins.

Definition of condensed tannins and their deposition in plant tissues

Condensed tannins (CT) are a complex group of phenolic compounds originated by the plant secondary metabolism and are also commonly known as proanthocyanidins (PA) (Waghorn, 2008). These polymers result mainly from the condensation of flavan-3-ol units ((epi)catechin and (epi)gallocatechin) which are linked by C-C bonds, the majority between C-4 and C-8 positions (Waghorn & McNabb, 2003; Patra & Saxena, 2011). Presenting a molecular weight up to 20 000 g/mol (Cieslak, Szumacher-Strabel, Stochmal & Oleszek, 2013), CT can be composed from two to twenty monomeric units with a variable degree of branching and hydroxylation patterns at several positions on each monomer (Waghorn & McNabb, 2003). A high diversity on molecular weight, chemical composition and molecular structure of CT occurs within and between plant species (Waghorn & McNabb, 2003) which is determinant to the reactivity and biological activity of CT and may explain the differences found on CT physiological effects and on animal performance (Waghorn, 2008). For instance, CT oligomers with low molecular weight are more reactive than those with higher molecular weight and CT degradation products originated by different monomeric units can also determine different biological efficacy (Patra & Saxena, 2011).

Condensed tannins are widespread through the plant kingdom, being the most common type of tannins present in forage legumes, shrubs, tree leaves, cereals and grains (Min, Barry, Attwood & McNabb, 2003; Patra & Saxena, 2011). On the other hand, their presence in the foliage of grasses is very low or they are even absent (Waghorn & McNabb, 2003). The accumulation of CT occurs into the cell wall or into intracellular inclusions of the vegetal tissues and is generally higher in the vulnerable parts of the plant, such as new leaves and

reproductive structures (flowers, seeds) (Patra & Saxena, 2011). The deposition of CT is mainly regulated by genetic factors (McMahon *et al.*, 2000), but environmental conditions such as temperature, light intensity, soil composition and quality, water stress and topography also exert an important influence (McMahon *et al.*, 2000; Patra & Saxena, 2011; Guerreiro *et al.*, 2015). High temperatures associated to low-fertility acidic soils are also being related to higher accumulation of CT in plant tissues (McMahon *et al.*, 2000). Moreover, under browsing by herbivores, some plants immediately increment their CT content in tissues (McMahon *et al.*, 2000).

Although the exact role of CT in plant metabolism is not fully understood, there are several hypotheses including anti-herbivory mechanism, defense against plant pathogens, protection against UV light, allelopathy, conservation of nitrogen and energy (McMahon *et al*., 2000; Waghorn, 2008). During storage into intracellular vacuoles, CT are essentially unreactive however, with the rupture of those structures during chewing, CT are released and when in contact with proteins, carbohydrates, lipids, cations and metal ions occurs an extensive binding between CT and those molecules, resulting in the formation of insoluble complexes (McMahon *et al.*, 2000; Waghorn & McNabb, 2003). Due to the higher affinity of CT to bound with proteins than with other molecules (Hagerman & Butler, 1981), CT-protein complexes are the mostly formed. Those can result from the complexation of CT with the protein of the feed components, of the animal (salivary, digestive and endogenous proteins) and of the rumen microorganisms (enzymes and structural proteins) (Waghorn, 2008). Astringency, which measures the protein binding capacity with CT, is higher for proline rich proteins (PRP) (Hangerman & Butler, 1981). It is the case of the salivary proteins of browsing ruminants, as goats, which in general contains higher concentrations of PRP comparing to those of grazers, as sheep (Rochfort, Parker & Dunshea, 2008; Waghorn 2008; Patra & Saxena, 2011). This specific response to dietary CT by different ruminant species constitutes a defense mechanism developed by browsers, which, due to their feeding habits, are more expose to the biological effects of a higher ingestion of CT. The formation of complexes between CT and the salivary enzymes in the mounth of the animal will significantly reduce the level of CT that will be ingested in an active form. This reduces *a priori* the effects that the ingested CT may exerce on rumen microbiota and on animal organism.

Effects of condensed tannins in ruminants

The biological effects of CT in ruminants may be perceptive in rumen metabolism, digestion, health and/or productive performance (Waghorn & McNabb, 2003; Rochfort *et al.*, 2008; Waghorn, 2008). The beneficial or the detrimental effects of inclusion of CT in ruminant diets is conditioned by different aspects as the concentration, the chemical nature of tannin, the

composition of the diet, the animal species and physiological status (McMahon *et al.*, 2000; Makkar *et al.*, 2003; Rochfort *et al.*, 2008). When CT are present at high concentrations in plant tissues (>6% DM) (Aerts, Barry & McNabb, 1999; Butter, Dawson & Buttery, 1999), normally exert anti-nutritive and toxic effects in ruminants. Those effects range from toxicosis and death to a reduction of voluntary feed intake and of protein digestibility, with consequences on the rate of amino acids absorption from intestine (Rochfort *et al.*, 2008; Waghorn & McNabb, 2003; Waghorn, 2008). Nevertheless, when ingested in balanced doses, CT may present confirmed benefits preventing bloat, excessive protein rumen degradation or acting as anthelmintics and as antioxidants, improving animal health and performance (Douglas, Stienezen, Waghorn, Foote & Purchas, 1999; McMahon *et al.*, 2000; Makkar, 2003; Min & Hart, 2003; Waghorn, 2008; Krueger *et al.*, 2010; Patra & Saxena, 2011; Brogna *et al.*, 2014).

Effects of condensed tannins on ruminant lipid metabolism and meat fatty acid composition

Condensed tannins and dietary tanniferous sources, may interfere with the last reductive step of RBH, increasing the accumulation of 11*t* -18:1 in the rumen and thus, have been proposed as potential modulators of RBH (Vasta & Bessa, 2012). The effects of CT on rumen microorganisms depend mostly of the microbial species and of concentration and characteristics of CT molecule (Patra & Saxena, 2011). The antimicrobial activity of CT can occur at the level of the structure of microbial cells, due to the interactions with the proteins of cell wall and membrane, causing morphologic changes of cell wall and disruption of microbial cellular membrane. Also, due to the formation of complexes with dietary components, CT reduces the substrates for microbial growth and metabolism and binding with microbial enzymes CT may inhibit the activity of microbial extracellular enzymes and induce alterations on the intracellular metabolism (Rochfort *et al.*, 2008; Patra & Saxena, 2011). The effects of CT on accumulation of *trans*-18:1 isomer in the rumen as been proposed to be due to a specific effect of group B biohydrogenating bacteria. A selective activity of CT, in reducing the population of *B. proteoclasticum* which performs the last step of RBH has been demonstrated (Sivakumaran *et al.*, 2004; Vasta, Makkar, Mele & Priolo, 2009a; Vasta *et al.*, 2010a; Khiaosa-Ard *et al.*, 2009). Sivakumaran *et al.* (2004) in an *in vivo* study with pure cultures demonstrated that the growth of *B. proteoclasticum* was inhibited by CT from *Dorycnium rectum* forage, independently of the CT molecular weight and the concentration of CT in the medium. However, the growth of *B. fibrisolvens* only was affected by the fractions of CT with low and medium molecular weight and independently of the levels of CT in the medium. Moreover, the fraction with higher molecular weight stimulated the growth of *B. fibrisolvens* in the medium with the lowest concentration of CT (100mgL^{-1}).

Vasta *et al.* (2010b) observed in lambs, that the inclusion of 9.6% of dry matter of quebracho tannin to a diet composed by a barley-based concentrate and lucern hay, increased the population of *B. fibrisolvens* while reduced the growth of *B. proteoclasticum*. Moreover, animals fed with tannins presented a higher content of 11*t*-18:1 in the rumen compared to those not supplemented with tannins. Also Khiaosa-Ard *et al.* (2009) using Rusitec system, reported that the addition of 78.9gkg^{-1} of dry matter of CT inhibited the conversion of 11*t*-18:1 to 18:0 in the BH of 18:3 n-3. Although *in vitro* studies shown that CT can induce changes in rumen microbial populations leading to positive effects on RBH resulting in accumulation of 11*t*-18:1 in rumen, the results from *in vivo* studies sometimes are not so clear. Vasta *et al.* (2009b) including 4% of quebracho tannins in herbage- or concentrate-based diets fed to lambs, reported that the addition of tannins to the concentrate-based diet increased 11*t*-18:1 and reduced 18:0 in the ruminal fluid and increased the concentration of 9*c*,11*t*-18:2 on meat. Jerónimo *et al.* (2010b) and Jerónimo *et al.* (2012) feeding lambs with dehydrated lucerne, also reported a significant increase of *t*11-18:1 and *c*9,*t*11-18:2 in lamb meat lipids with the inclusion of 25% *Cistus ladanifer*, but only when diet was also supplemented with 6 % of a oil blend of sunflower and linseed oils (1:2 v/v). Whitney *et al.* (2011) including red berry juniper leaves in feedlot growing rations of lambs, observed that the proportion of 9*c*,11*t*-18:2 in meat increased while 18:0 decreased. Rana *et al.* (2012) feeding kids with concentrate supplemented with aqueous extract of *Terminalia chebula*, reported, in the rumen fluid, a reduction of 18:0 and an increase in 11*t*-18:1 with the higher supplementation level (6 mg/ml of rumen volume), suggesting a interference of *T. chebula* extract on the last step of RBH and thus higher content of 11*t*-18:1 escaping from rumen as observed from the fatty acid profile of plasma.

The influence of dietary CT in the regulation of endogenous synthesis of 9*c*,11*t*-18:2 by Δ9-desaturase has been suggested. When included quebracho tannins in concentrate and herbage-based diets for lambs (Vasta *et al.*, 2009b), an increase on Δ-9 desaturase protein expression in the case of the herbage-based diet was observed (Vasta *et al*., 2009c). Rana *et al.* (2012) also reported higher activity of Δ-9 desaturase in the microsomes of muscle of kids supplemented with *T. chebula* that result in a higher production of 9*c*,11*t*-18:2 from endogenous conversion of 11*t*-18:1 by the action of Δ9-desaturase.

Effects of condensed tannins on meat organoleptic quality and oxidative stability

Feeding plant secondary compounds as CT to small ruminants can influence some meat quality parameters (Vasta *et al.*, 2008; Vasta & Luciano, 2011). The inclusion of different tanniferous plant species in lamb diets has been associated to the increase of meat lightness (Priolo, Lanza, Biondi, Pappalardo & Young, 1998; Priolo, Waghorn, Lanza, Biondi & Pennisi,

2000; Lanza *et al.*, 2001; Priolo, Ben Salem, Atti & Nefzaoui, 2002b; Priolo *et al.*, 2005). However, that effect of dietary CT on meat lightness seems to be dependent of the type of CT source, since the lack of influence on colour parameters by CT is also reported (Jerónimo *et al.*, 2012). In order to correspond to the increasing concerns of consumers about the addition of synthetic feed additives to increase food shelf life, including meat and meat products, the interest of industry for plants and plant extracts as sources of natural antioxidants has been increasing in the last few years. In that context, it is being reported that the addition of CT or tannins sources to the diet of ruminants is a successful strategy to increase meat colour and lipid stability (Luciano *et al.*, 2009b; 2009c; Luciano *et al*., 2011b; Jerónimo *et al.*, 2012).

The inclusion of a tanniferous bush (Cistus ladanifer, L.*) in the diet of ruminants to improve meat lipid composition*

Cistus ladanifer L. (*C. ladanifer*), commonly known by rockrose, is an evergreen spontaneous aromatic shrub which is well adapted to poor and acidic soils and semi-arid conditions being abundant in marginal fields of most Mediterranean countries, where it can form vast shrublands (Patón, Azocar & Tovar, 1998; Robles, Bousquet-Mélou, Garzino & Bonin, 2003) *Cistus ladanifer* presents high levels of plant secondary metabolites, such as polyphenolic compounds, phenolic acids, CT and other flavonoids and also terpenic compounds (Chaves Escudero & Gutiérrez-Merino, 1997; Dentinho, Navas & Potes, 2005; Gomes, Mata & Rodrigues, 2005; Barrajon-Catalan *et al.*, 2010; Barros *et al.*, 2013; Guerreiro *et al*., 2016). Recently, several *in vitro* studies and laboratorial assays conducted with *C. ladanifer* demonstrated its antibacterial, antifungal, antioxidant, and antitumoral activities (Andrade, Gil, Breitenfeld, Domingues & Duarte, 2009; Greche *et al.*, 2009; Barrajón-Catalán *et al.*, 2010; Ferreira *et al.*, 2012; Barros *et al.*, 2013). Although with low nutritive value and low acceptability by grazing ruminants (Dentinho *et al.*, 2005), *C. ladanifer* can be included up to 25% DM in high forage diets (Jerónimo *et al.*, 2010b, 2012), without negative effects in growth performances, carcass and meat quality attributes. Moreover, dietary *C. ladanifer* is able to decrease the meat lipid oxidation (Jerónimo *et al.*, 2012). When used together with vegetable oils, *C. ladanifer* enhanced the effects of oil inclusion on the accumulation of biohidrogenation intermediates, indicating that it has a direct impact in RBH, improving the deposition of 11*t*-18:1 and 9*c*,11*t*-18:2 in lamb meat (Jerónimo *et al.*, 2010b). In that experiment it was demonstrated that *C. ladanifer* has potential to modulate RBH, at least in high forage basal diets. Commercial lamb finishing diets are mostly based on concentrates, thus it is very pertinent to explore if the effects of *C. ladanifer* on RBH are maintained with concentrate-based diets.

Figure 1.16 - *Cistus ladanifer* L. (common name: gum rockrose, labdanum (EN); esteva (PT)). Pictures from M.T. Dentinho

1.6. Objectives

The main objective of the present study was the development of three possible nutritional strategies to promote the enrichment on vaccenic (11*t*-18:1) and rumenic (9*c*,11*t*-18:2) acids, as well as n-3 LC-PUFA on meat from lambs fed with forage-concentrate basal diets (50:50).

Therefore, two *in vivo* trials were conducted according to the main drives presented on Figure 1.15 and with the specific objectives:

Experiment 1- Inclusion of increasing levels of a tanniferous bush (*Cistus ladanifer* L.) and of a vegetable oil blend composed by linseed and soybean oils (2:1 vol/vol) in the diets of lambs from intensive feeding system:

- Evaluate the effects of the dietary inclusion of increasing levels of *Cistus ladanifer* (Cistus) (5, 10 and 20 % DM) and of oil supplementation (0, 4 and 8 % DM) on growth performance, carcass composition and meat overall quality of lambs (**Chapter 2**).

- Evaluate the effects of the interaction between Cistus x Oil dietary inclusion on the proportions of 11*t*-18:1, 9*c*,11*t*-18:2 CLA and n-3 LC-PUFA of intramuscular fat and on the gene expression of Δ9-,Δ6- and Δ5- desaturases (**Chapter 3**).

Experiment 2- Inclusion of *Cistus ladanifer* L. and inclusion of dehydrated citrus pulp (DCP) relacing cereals, in high-soybean oil diets of lambs from intensive feeding system:

- Evaluate the effects of the experimental diets on lamb´s productive performance, carcass traits and meat quality, including meat oxidative stability and sensory attributes (**Chapter 4**).

- Evaluate if the replacement of cereals by DCP and the combination of DCP and *Cistus ladanifer* may promote the enrichment of 11*t*-18:1 and of 9*c*,11*t*-18:2 in the meat from lambs fed with concentrate-based diets supplemented with 6% of soybean oil (**Chapter 5**).

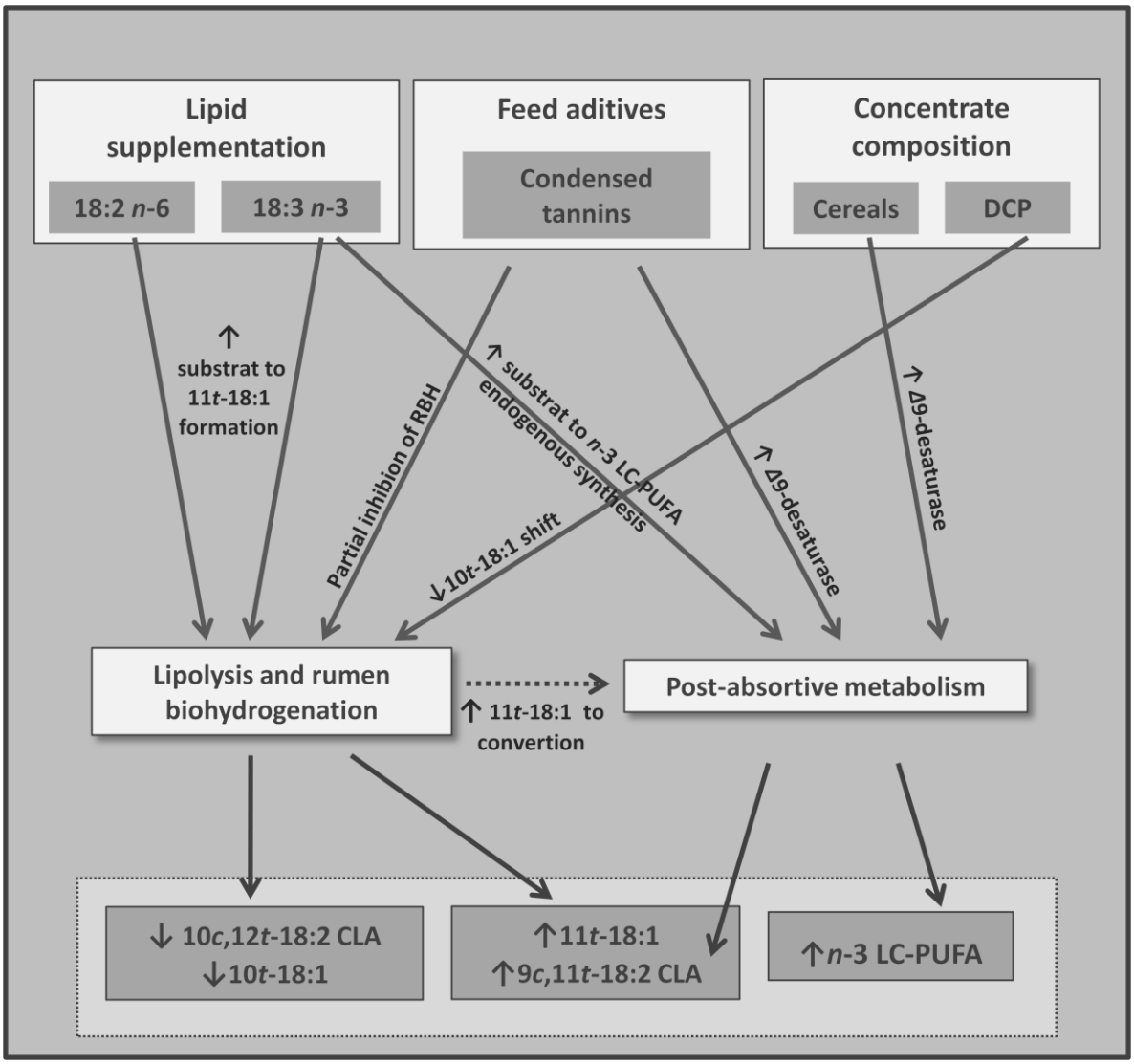


Figure 1.16 – Nutritional strategies and related main drives involved in the design of the present study.

CHAPTER 2

Growth performance, carcass and meat quality of lambs supplemented with increasing levels of a tanniferous bush (*Cistus ladanifer* L.) and vegetable oils

Growth performance, carcass and meat quality of lambs supplemented with increasing levels of a tanniferous bush (*Cistus ladanifer* L.) and vegetable oils

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Contribution of Alexandra E. Francisco to this article:

Alexandra E. Francisco participated on the peleting of the diets, in the animal experiment and in the sample collection. Determined the fatty acid content, collaborated on the evaluation of meat quality parameters and performed the data processing and statistical analysis. Furthermore, Alexandra E. Francisco integrated the trained sensory analysis panel and participated in the interpretation and discussion of the results, as well as in the writing of the manuscript.

ABSTRACT

The effects of dietary inclusion of *Cistus ladanifer* L. (Cistus) and a vegetable oil blend were evaluated on growth performance, carcass and meat quality of fifty four lambs that were assigned to 9 diets, corresponding to 3 levels of *C. ladanifer* (50, 100 and 200 g/kg DM) and 3 levels of oil inclusion (0, 40 and 80 g/kg DM). Treatments had no effects on growth rate. Oil depressed dry matter intake ($P = 0.017$), carcass muscle ($P = 0.041$) and increased ($P = 0.016$) kidney knob channel fat. Chemical and physical meat quality traits were not affected by treatments. Off-flavour perception was higher for 8% of oil ($P < 0.001$). The level of 100 g/kg DM of *C. ladanifer* inclusion improved meat stability after 7 days of storage. Supplementation with linseed and soybean oils (2:1) was a good approach to improve meat nutritional value from feedlot lambs, increasing total n-3 PUFA.

Key words: Lamb, *Cistus ladanifer*, oil supplementation, carcass quality, meat quality

2.1. Introduction

Ruminant edible fat contains high levels of saturated fatty acids (SFA), low contents of polyunsaturated fatty acids (PUFA) and variable amounts of rumen metabolism derived fatty acids (FA), including *trans* FA and conjugated FA. Increasing meat's content of PUFA (particularly *n*-3 PUFA) and conjugated linoleic acid isomers (CLA) is widely accepted as targets to improve nutritional quality of ruminant meat (Mapiye *et al.*, 2012). Dietary supplementation with PUFA rich oils can be used to increase both *n*-3 PUFA and CLA, but the extensive rumen biohydrogenation of PUFA strongly limits the effectiveness of this nutritional strategy.

Cistus ladanifer L. is an evergreen spontaneous forage shrub well adapted to semi-arid conditions, poor and acidic soils (Patón *et al.*, 1998) and highly available in marginal fields of most Mediterranean countries. Overall, the *C. ladanifer* is considered as a feed resource with low nutritional value, mainly due to its high content on antinutritional compounds, such as condensed tannins (CT) (Dentinho *et al.*, 2005), being a common field observation that ruminants only browse *C. ladanifer* during season of pasture scarcity. Condensed tannins are normally considered as anti-nutritive and toxic compounds due to their adverse nutritional effects for herbivores, especially when they are in a high concentration in plant tissues (>6% DM) (Aerts *et al.* 1999; Butter *et al.*,1999). Nevertheless, when ingested in balanced doses, the CT may have beneficial effects on ruminants by preventing bloat, increasing digestive utilization of dietary protein, and acting as anthelmintics and as antioxidants (Makkar, 2003; Waghorn, 2008). Condensed tannins might have some potential to interfere with rumen

metabolism and modulate the PUFA biohydrogenation, as well as to improve the meat oxidative stability (Vasta & Luciano, 2011). Thus, our research team explored the possibility of achieving these goals by incorporating *C. ladanifer* in high oil-high forage diets of finishing lambs (Jerónimo *et al.*, 2010b, 2012). In the previous studies we reported that *C. ladanifer* modified the rumen biohydrogenation pattern, resulting in a relevant enrichment of CLA in intramuscular fat of oil supplemented lambs (Jerónimo *et al.*, 2010b). In addition, *C. ladanifer* increased the resistance to induced oxidation in meat, independently of oil supplementation (Jerónimo *et al.*, 2012). Moreover, we were surprised by the fact that the inclusion of *C. ladanifer* up to 25% dry matter (DM) did not affect negatively growth, carcass traits and meat quality of lambs (Jerónimo *et al.*, 2010b, 2012). In that study, *C. ladanifer* replaced dehydrated lucerne in high forage diets and this might be a reason why its inclusion did not results in productive traits depression. However, finishing lambs are commonly fed low forage diets based on cereals, cereals by-products and oilseed meals. Thus, the present experiment was designed to test if the *C. ladanifer* effects observed when *C. ladanifer* is included in high oil-high forage diet, are maintained when *C. ladanifer* is included in diets with lower forage content. The productive performance, carcass traits and meat quality results are reported here.

2.2. Materials and methods

2.2.1. Animals and management

Animal handling followed EU Directive 86/609/EEC concerning animal care. Fifty-four Merino Branco (MB) ram lambs born in spring 2012 were reared with dams on extensive grazing until weaning at approximately 60 days of age. At weaning, lambs were transported to the Unidade Estratégica de Investigação em Produção e Saúde Animal, Instituto Nacional de Investigação Agrária e Veterinária (UEIPSA-INIAV), located at Vale de Santarém, Portugal. Thereafter, lambs were housed and randomly assigned to 18 pens; 3 lambs per pen and 2 pens per treatment, according to a completely randomized experimental design with a 3×3 factorial arrangement of treatments, with 3 levels of Cistus incorporation (50, 100 and 200 gDM / kgDM) and 3 levels of oil (0, 40 and 80 g/kg), resulting in 9 isoenergetic and isonitrogenous diets: 1) Cistus5, 5% *C. ladanifer* and 0% oil; 2) Cistus10, 10% *C. ladanifer*and 0% oil; 3) Cistus20, 20% *C. ladanifer* and 0% oil; 4) Cistus5O4, 5% *C. ladanifer* and 4% oil; 5) Cistus10O4, 10% *C. ladanifer* and 4% oil; 6) Cistus20O4, 20% *C. ladanifer* and 4% oil; 7) Cistus5O8, 5% *C. ladanifer* and 8% oil; 8) Cistus10O8, 10% *C. ladanifer*and 8% oil; 9) Cistus20O8, 20% *C. ladanifer* and 8% oil. Leaves and soft stems of Cistus shrubs were harvested in Portugal (39°30'36"N/8°19'00"W) in March 2012, dried at room temperature, cut in small pieces, and milled. The oil used as supplement was a blend of

soybean oil and linseed oil (1:2 vol/vol). The diets were presented as pellets (3 mm diameter). The formulas of the 9 diets and their chemical composition, obtained as the average of the results of three pooled samples of each diet, are presented in Table 2.1.

After an adaptation period of 7 days to the experimental conditions, in which lambs were dewormed by dosing with Ivomec® (Merial Labs., Spain) and vaccinated against enterotoxaemia (Miloxan, Merial Labs., Spain), the lambs stayed on trial for 6 weeks. The average live weight (LW) at the beginning of the trial was 16.2±2.93 kg (mean±SD). Feed was offered daily at 9:00 am at a rate of 110% of *ad libitum* intake calculated by weighing-back refusals daily which were registered and discarded. The animals were weighed weekly just before feeding.

Table 2.1 – Proximal (%) and chemical composition (g/kg DM) of the experimental diets

	5 Cistus			10 Cistus			20 Cistus		
	0 Oil[a]	4 Oil	8 Oil	0 Oil	4 Oil	8 Oil	0 Oil	4 Oil	8 Oil
Ingredients									
Maize	5.00	5.00	5.00	26.11	21.34	16.57	23.91	19.71	5.00
Wheat	29.55	24.42	19.30	6.00	6.00	6.00	6.62	6.00	16.68
Soybean meal 48%	12.95	14.08	15.20	15.39	16.16	16.93	16.98	17.79	17.82
Cistus ladanifer	5.00	5.00	5.00	10.00	10.00	10.00	20.00	20.00	20.00
Dehydrated lucerne	45.00	45.00	45.00	40.00	40.00	40.00	30.00	30.00	30.00
Oil[a]	0.00	4.00	8.00	0.00	4.00	8.00	0.00	4.00	8.00
Sodium bicarbonate	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Calcium carbonate	1.30	1.30	1.30	1.30	1.30	1.30	1.30	1.30	1.30
Minerals and	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Salt	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Chemical									
Dry matter (g/kg)	910	915	918	910	916	917	910	912	916
Crude protein	165	157	164	162	156	159	165	162	155
NDF [b]	368	374	364	361	344	354	334	320	316
Starch	255	249	258	263	241	198	258	222	180
Ether extract	23	63	105	30	70	112	38	78	116
Total phenols	5.9	5.8	7.6	10.5	10.0	12.6	20.0	21.5	22.2
CT[c]	2.5	2.9	2.6	6.5	6.9	7.4	16.3	14.5	16.1

[a] - soybean oil and linseed oil (1:2 vol/vol), [b] - neutral detergent fiber; [c] - condensed tannins

2.2.2. Slaughter, carcass evaluation and sample collection

At the end of the trial, lambs were weighed and transported to the experimental abattoir of the UEIPSA-INIAV where they were stunned and slaughtered, by sectioning jugular veins and the carotid arteries. After preparation, carcasses were immediately weighed to obtain hot carcass weight (HCW) and were kept at 10 °C for 24h. After that period carcasses were re-weighed, to

obtain the cold carcass weight (CCW) and graded, according to the EUROP classification systems for lamb carcasses weighing less or more than 13 kg (EC regulations Nº1234/2007 and Nº1249/2008 (European Commssion [EC], 2011a, 2011b)). Then, carcasses were chilled at 2 °C until the third day after slaughter. At that time, the kidney knob channel fat (KKCF) and kidneys were removed. Carcasses were split along the spine and left sides were separated into eight joints as described in Santos-Silva, Mendes and Bessa (2002b). The weight of each joint was recorded to estimate the proportion of the higher-priced joints (leg + chump + loin + ribs). The pH was measured in the *Semimembranosus* muscle (SM) using a Hanna Instruments Hi 9023 device. Chumps and shoulders were vacuum-packed and frozen at -20 °C until being dissected into muscle, subcutaneous and intermuscular fat and bone to determine the tissue composition.

The rib joints of the left half of the carcasses, containing the *Longissimus thoracis* muscle (LT), were vacuum-packed and frozen at -20 °C until shear force determinations. The *Longissimus lumborum* (LL) muscle was removed from loin joints of the left halves of the carcasses and, after the removal of the *epimysium*, was minced with a food processor (3×5 s), vacuum-packed, freeze-dried, and stored at -20 °C until further lipid analysis.

In the right halves, two sub-samples with about 1.5 cm thickness of LT were collected and used to evaluate the lipid and colour stability during 7 days of storage at 2 °C in illuminated cooler. At day 0 of storage the colour parameters were determined after 1 hour of blooming. The other sample was individually placed on polystyrene trays, over-wrapped with oxygen permeable polyvinyl chloride film and displayed 7 days. At the end of storage time, meat samples were allowed to bloom for 1 h, before the determination of the colour parameters. After that, the samples were vacuum packed and stored at -80 °C until lipid oxidation analysis. The right loins, containing LL were vacuum packed and frozen at -20 °C, until being used for sensory analysis.

2.2.3. Analytical procedures

2.2.3.1. Feed and muscle chemical analysis

Diets were analysed for DM (ISO 6496, 1999), crude protein (ISO 5983, 1997), ether extract (EE) (ISO 6492, 1999). Neutral detergent fibre (NDF), which was assayed according to Van Soest, Robertson, and Lewis (1991) with sodium sulphite, without alpha amylase and expressed with residual ash method and starch was determined according to Clegg (1956). Fatty acid methyl esters (FAME) of feed lipids were prepared by a one-step extraction transesterification with toluene and heptadecanoic acid (17:0) as internal standard, according to Sukhija and Palmquist (1988).

The extraction of phenolic compounds was carried out as described by Khazaal, Markantonatos, Nastis, and Orskov (1993). The extracts obtained were used for determination of total phenols and CT. Total phenols were determined by Folin–Ciocalteu's reagents, according to Julkunen-Tiito (1985) and the concentration was measured as tannic acid equivalent using tannic acid as standard. Total extractable CT were measured using butanol-HCl method (Porter, Hrstich & Chang, 1986). The concentration of CT in the diets were quantified using CL purified CT as standard.

Intramuscular lipids were extracted according to Folch, Lees and Stanley (1957) method, as described by Jerónimo *et al.* (2009). Fatty acid methyl esters were analysed using a HP6890A chromatograph (Agilent, Avondale, PA, USA), equipped with a flame-ionization detector and fused silica capillary column (SP-2560 (100m x 0.25mm internal diameter x 0.20um film thickness, Supelco, Bellefonte, PA, USA)). The injector and detector temperatures were 250 and 280 °C, respectively. Initial oven temperature of 100 °C was held for 1 min, increased at 50 °C/min to 150 °C and held for 20 min, increased at 1 °C/min to 190 °C and held for 5 min, and then increased at 1 °C/min to 200 °C and held for 35 min. Helium was used as carrier gas at a flow rate of 1 ml/min, the split ratio was 1:30 and 1 ul of sample was injected. Quantification of muscle lipid FA methyl esters was done using nonadecanoic acid (19:0) as internal standard. Fatty acid composition is presented as partial sums of selected fatty acids expressed as mg/100 g of meat.

2.2.3.2. Measurement of shear force

For shear force determinations, the frozen left ribs were thawed for 24 h at 2 °C, LT muscle was isolated and cooked in an electric oven at 280 °C until the meat internal temperature reached 70 °C. Then, after cooling for 1 h, each muscle sample was cut in the direction of the fibres, in subsamples with a section of about 1 cm^2 and 2 cm long. Shear force (kg) was determined using a Warner-Bratzler shear device, mounted in a Texture Analyser (TA-tx2i Texture Analyser, Stable Micro Systems, Surrey, UK). It was used a compression load cell of 25 kg and a crosshead speed of 2 mm/s, along 25 mm. Data were collected using specific software (Texture Expert Exceed, Stable Micro Systems, Surrey, UK). The measurement of cores from each loin was recorded as the average of a minimum of six subsamples, of the maximum force needed to shear the samples perpendicularly to the axis of the fibre direction.

2.2.3.3. Measurement of meat colour

The colour of the LT samples was measured using a Minolta CR-300 Chromometer (Konica Minolta, Portugal) according to CIE *L**, *a**, *b** system, where *L** is lightness, *a** redness and *b** yellowness. Measurements were made from a 2 º - viewing angle and using illuminant C. Hue angle (H*) was calculated as $\tan^{-1}(b^*/a^*) \times (180/\pi)$ and colour saturation (chroma, C*) as $(a^{*2} + b^{*2})^{1/2}$. The effect storage on meat colour was evaluated by the indexes $\Delta E_{(7-0)}=((\Delta L^*_{(7-0)})^2+(\Delta a^*_{(7-0)})^2+(\Delta b^*_{(7-0)})^2)^{0.5}$, $\Delta H_{(7-0)}$= (Hue value day 7) – (Hue value day 0) and $\Delta C_{(7-0)}$= (Chroma value day 7) – (Chroma value day 0).

2.2.3.4. Measurement of lipid oxidation

Lipid oxidation in meat was assessed through the quantification of thiobarbituric acid reactive substances (TBARS), following the method described by Grau, Guardiola, Boatella, Barroeta and Codony (2000). Briefly, 2 g of meat were homogenized in 1 mL of 0.3% aqueous ethylenediaminetetraacetic acid disodium salt (EDTA) , 8 ml of 5% aqueous trichloroacetic acid (TCA) and 5 mL of 0.8 % butylated hydroxytoluene (BHT) in hexane using an Ultra-Turrax T25 digital homogenizer (IKA Werbe GmbH & Co. KG, Staufen, Germany) for 30 s at 19 000 rpm. The homogenates were centrifuged during 5 min at 1400 g and the top hexane layer discarded. The bottom layer was filtered, and to filtrate was added TCA (5% aqueous) to make up a volume of 10 mL. Aliquot of 2.5 ml from the bottom layer was mixed with 1.5 ml of 0.8% aqueous 2-thiobarbituric acid (TBA) and incubated at 70 °C for 30 min. Following incubation, the mixture was cooled under tap water and the absorbance was measured at 532 nm in a Double-beam UV-Vis scanning spectrophotometer (Helios alpha spectrophotometer, Thermo Scientific, Bremen, Germany). The 1,1,3,3 tetraethoxypropane standard curve was used for calculating the TBARS concentration and the results were expressed as mg of malonaldehyde (MDA)/kg of meat.

2.2.3.5. Sensory analysis

Sensory meat quality was evaluated in six sessions by a trained sensory panel with eleven members. For each session, 9 frozen loin joints were randomly selected and thawed for 24 h at 7 °C. The LL muscle was isolated and cooked in a plate grill (65/70 FTES Electric Griddle, Modelar Catering Equipment, Italy) at 250 °C, until they reached an internal temperature of 71 °C, which was monitored by an internal thermocouple (Lufft C120, München, Germany). Cooking losses, used as a measure of meat water holding capacity, were determined as the difference in meat sample weights before and after cooking, and expressed as a percentage of initial weights. Every LL sample was then trimmed of any external connective tissue and

cut into 1×1×1 cm^3 samples and maintained at 60 °C in heated plaques, until tasting. The attributes retained were tenderness, juiciness, flavour and overall acceptability. The scale applied in the sensory analysis was structured into eight points, where: 1- extremely tough, extremely dry, weak or extremely negative and 8- extremely tender, extremely juicy, strong or extremely positive. If there were present any off-flavours (≥ 1 point score), they were identified and registered.

2.2.4. Statistical analysis

Data were analysed as completely randomized experimental design following a 3×3 factorial treatment arrangement using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) where the mains effects and its interaction was included in the model. When not significant, the interaction was removed from the model. Animal with pens were treated as non independent replicates and considered as repeated measure within pens assuming a compound symmetry covariance matrix.

Daily weight gain (ADG) was determined individually by linear regression analysis. Data from meat sensory evaluation were analyzed with the MIXED procedure of SAS considering each observation from each panellist as the repeated measurement within assuming a compound symmetry covariance matrix.

Because most of the interactions were not significant, the tables only present the LSMeans concerning the main effects and the standard error of means (SEM). The level of statistical significance was set at $P<0.05$.

2.3. Results

2.3.1. Growth performance and carcass traits

The results concerning growth performance are presented in Table 2.2. The growth rate of lambs and feed intake to weight gain ratio were not affected by treatments and the average values were 267±42.6 g and 5.7±0.62 respectively. Oil supplementation depressed the dry matter intake (DMI) (P=0.017). The feed intake observed for 0% oil were higher (120 g/kg $LW^{0.75}$) than those observed for 8% oil inclusion (109 g/kg $LW^{0.75}$).

Hot and cold carcass weights and dressing percentage results were not affected by treatments, averaging 14.9±0.80 kg, 14.5±0.79 kg and 46.2±0.73% respectively.

Table 2.2. - Effects of *Cistus ladanifer* (Cistus) and oil supplementation (Oil) on growth performance, carcass quality and chump and shoulder composition of Merino Branco lambs

	Cistus ladanifer			**Oil**			**SEM**	***P* value**	
	5	**10**	**20**	**0**	**4**	**8**		**Cistus**	**Oil**
Slaughter weight (kg)	31.8	32.6	32.9	31.2	33.6	32.5	1.31	0.829	0.428
DMI (g/d/kg $wt^{0.75}$)	110	119	115	120^{b}	115^{ab}	109^{a}	4.0	0.291	0.017
ADG (g/d)	255	300	251	259	279	267	19.8	0.192	0.771
Feed/weight gain ratio	5.75	5.57	5.87	5.73	5.20	6.25	0.311	0.792	0.094
Hot carcass weight (kg)	14.5	15.1	15.4	14.4	15.4	15.2	0.75	0.715	0.595
Cold carcass weight (kg)	14.1	14.6	14.9	13.9	15.0	14.7	0.75	0.732	0.563
Dressing (%)	45.6	46.2	46.6	46.1	45.8	46.5	0.63	0.556	0.749
Carcass conformation	2.11	2.43	2.53	2.38	2.28	2.42	0.174	0.236	0.844
Fat cover	1.94^{a}	2.38^{b}	2.61^{c}	2.33	2.22	2.39	0.167	0.034	0.760
HPJ (%)	54.1	54.0	53.8	54.3	54.0	53.5	0.23	0.625	0.064
Muscle (%)	60.1	59.6	59.1	60.5^{a}	60.0^{ab}	58.4^{b}	0.53	0.448	0.041
Bone (%)	20.0	19.7	18.9	20.0	19.7	18.9	0.55	0.365	0.386
Muscle/Bone ratio	3.02	3.08	3.16	3.06	3.08	3.12	0.091	0.578	0.880
Subcutaneous fat (%)	9.26	9.98	11.0	9.14	9.85	11.2	0.732	0.286	0.173
Intermuscular fat (%)	9.35	9.48	9.78	9.02	9.36	10.2	0.524	0.840	0.279
KKCF (%)	1.90	2.21	2.45	1.81^{a}	2.18^{ab}	2.56^{b}	0.152	0.074	0.016

DMI - dry matter intake; ADG - average daily weight gain; HPJ – higher price joints; KKCF – kidney knobb channel fat

Only three carcasses had weights lower than 13 kg and were not graded. The conformation scores were not affected by treatments (Table 2.2) and 40.8% of the lamb carcasses were graded as class R (good), 53.1% as O (regular) and 6.1% as P (poor) (data not shown). However, the fat cover scores increased with Cistus level, from 1.94 (5% Cistus) to 2.38 (10% Cistus) and 2.61 (20% Cistus). Figure 2.1 presents the distribution of the carcasses for fat cover classes. The increase of oil level had no clear effect on carcass distribution in the fattening classes, but, independently of the diet, most of the carcasses were graded in classes 2 and 3.

Oil supplementation decreased muscle percentage in chumps and shoulders (P=0.041) and increased kidney and knob channel fat (P=0.016) (Table 2.2). The proportion of higher priced inclusion was not affected by *C. ladanifer* inclusion in diets, but tended to decrease slightly with oil supplementation (P=0.064).

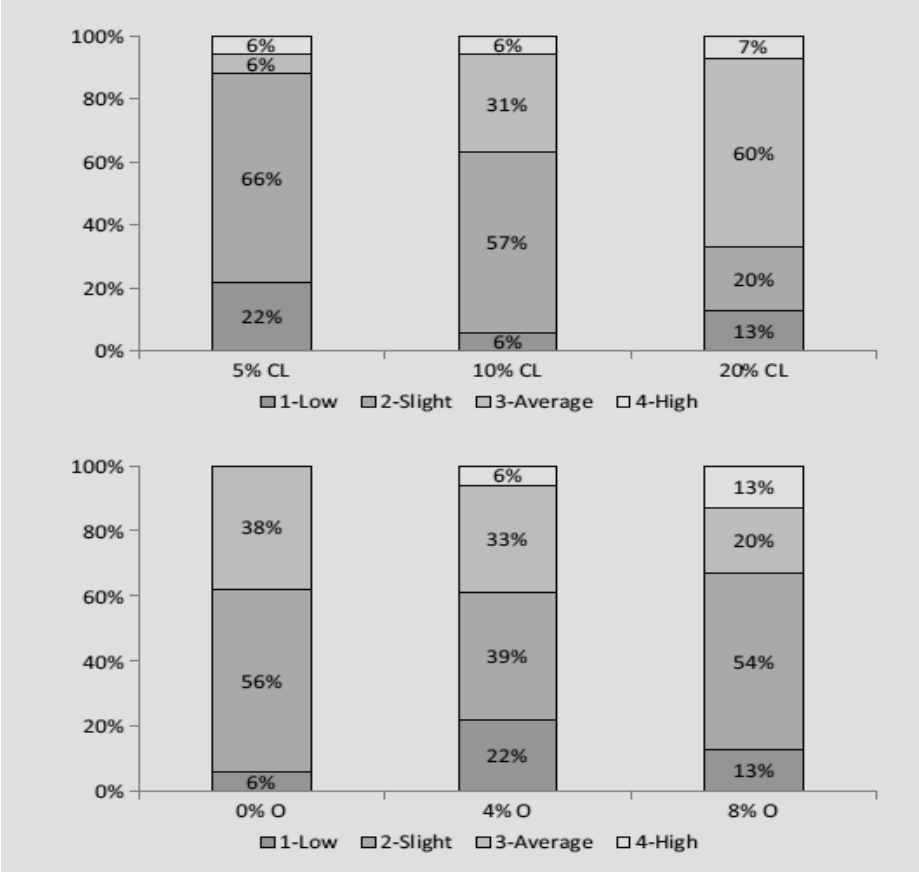

Figure 2.1 – Results of EUROP classification grading system for fat cover of carcasses weighing more than 13 kg (N=49) by level of *C. ladanifer* (CL) or oil inclusion in the diets

2.3.2. Meat quality parameters

Physical and sensorial attributes of lamb meat are presented in Table 2.3, as well as the indexes used to evaluate the colour variation and the lipid oxidation after 7 days of storage. The physical characteristics of meat were not affected by treatments. Average of meat colour parameters at day 0 of storage were 39.9±2.31 for *L**, 14.4±1.15 for *a** and 3.80±0.910 for *b**, cooking loss was 34.7±2.01% and shear force was 3.53±1.352 kg. However, for the colour indexes it was observed effect of the Cistus inclusion of diets, verifying a reduction of the ΔE(7-0), ΔHue(7-0) and ΔChroma(7-0) in meat of lambs fed diets with 10% or 20% of *C. ladanifer* comparatively with lambs receiving 5% of Cistus. The colour indexes were similar among lambs fed 10 and 20% of Cistus. The lipid oxidation of meat after 7 days of storage also was affected by Cistus inclusion of diets (*P*=0.024), observing lower lipid oxidation in meat from lambs fed with 20% of Cistus than in those receiving 5% of Cistus. The meat from

lambs fed with 10% of Cistus showed equal lipid oxidation than those fed diets with 5 and 20% of Cistus. Oil supplementation of diets did not affect either the colour indexes as the meat lipid oxidation.

Globally, meat was considered as moderately tender (6.2± 1.62) and the range of the values obtained within the treatments was similar, from 4.4±0.55 to 7.4±0.33. There was a significant Cistus × Oil interaction for tenderness (P=0.012) and juiciness (P<0.001). Oil inclusion depressed tenderness of meat from lambs fed with 5% Cistus. However, for 10% of Cistus the lowest value of tenderness was obtained for 4% of oil inclusion, while for 20% Cistus the effect of oil was not significant. Juiciness was depressed by oil inclusion for 5% and 10% of Cistus but for 20% the oil supplementation had no effect. The inclusion of *C. ladanifer* in the diet increased meat flavour (P=0.025). In spite of the very low scores for off-flavour intensity, it increased with *C. ladanifer* (35%, P=0.024) and oil (79%, P<0.001). In the 529 samples tested by the sensory panel it was recognized the presence of off-flavours in 158 of them (30% of the total) and the most frequent off-flavour identified and with higher intensity was livery (0.25±0.920). Overall acceptability was not affected by C. *ladanifer* inclusion. Group supplemented with 8% oil showed a higher score for off-flavour (P<0.001) and a lower overall acceptability (P=0.003).

Table 2.3 – Effects of *Cistus ladanifer* (Cistus) and oil supplementation (Oil) on physical, chemical and sensorial meat characteristics and in colour and lipid stability of Merino Branco lambs.

	Cistus ladanifer			**Oil**			**SEM**	***P* value**	
	5	**10**	**20**	**0**	**4**	**8**		**Cistus**	**Oil**
Meat pH	5.63	5.63	5.61	5.61	5.61	5.64	0.026	0.871	0.616
Cooking losses (%)	34.9	35.2	34.1	35.2	35.5	33.6	1.29	0.825	0.592
Shear force (kg)	3.25	3.57	3.87	3.53	3.90	3.26	0.397	0.572	0.533
Color parameters[1]									
*L**	39.5	40.1	40.2	40.3	39.9	39.5	0.52	0.572	0.588
*a**	14.5	14.3	14.4	14.5	14.2	14.4	0.31	0.906	0.837
*b**	3.85	3.70	3.85	4.04	3.62	3.73	0.269	0.904	0.516
Chroma	15.0	14.8	14.9	15.1	14.7	14.9	0.31	0.894	0.730
Hue angle	14.8	14.5	15.0	15.6	14.2	14.5	1.03	0.945	0.607
Color stability[2]									
$\Delta E_{(7-0)}$	8.33^{b}	6.52^{a}	5.93^{a}	6.35	6.59	6.84	0.507	0.006	0.094
$\Delta Hue_{(7-0)}$	31.4^{b}	24.2^{a}	21.6^{a}	23.4	24.2	29.6	2.25	0.013	0.112
$\Delta Chroma_{(7-0)}$	-2.79^{b}	-1.37^{a}	-1.03^{a}	-1.44	-1.20	-2.55	0.49	0.039	0.129
TBARS at day 7[3]	1.74^{b}	1.37^{ab}	0.96^{a}	1.19	1.37	1.62	0.163	0.024	0.220
Trained sensory evaluation									
Tenderness‡	6.38	6.26	6.08	6.41	6.11	6.21	0.109	0.171	0.140
Juiciness‡	3.58	3.88	3.70	3.91^{b}	3.84^{b}	3.41^{a}	0.110	0.120	0.003
Flavour intensity	3.71^{a}	4.16^{b}	3.92^{ab}	3.86	4.08	3.85	0.118	0.025	0.293
Off-flavour intensity	0.86^{ab}	0.73^{a}	1.15^{b}	0.74^{a}	0.67^{a}	1.34^{b}	0.113	0.024	<0.001
Overall acceptability	4.81	4.98	4.63	5.04^{b}	4.89^{b}	4.50^{a}	0.111	0.062	0.003

[1] at day 0 of storage; [2] rate of change after 7 days of storage;[3] mg MDA/kg of meat; TBARS – Thiobarbituric acid reactive substances; MDA – malonaldehyde; ‡ - Cistus× Oil interaction.

2.3.3. Meat nutritional value

Intramuscular fat content of LL and some relevant nutritional FA sums are presented in Table 2.4. Based on individual FA composition (results not shown), the content of SFA, *trans*-MUFA, *cis*-MUFA, PUFA, n-6 PUFA, n-3 PUFA and the long chain n-6 and n-3 PUFA (LC-PUFA i.e. C20 and C22) were reported to 100 g of fresh meat. Intramuscular fat content and total FA in meat were not affected by the treatments, and the average values were 2.07±0.41% and 1.26±0.158% respectively. Dietary *C. ladanifer* inclusion at 20% increased (*P*=0.014) the Total *trans*-MUFA. Oil supplementation increased the Total *trans*-MUFA (*P*=0.016) from the basal level of 42.6 mg/100 g of meat to 87.0 mg/100 g of meat with 8% of oil inclusion in the diet. The Total n-3 PUFA increased (*P*<0.001) with oil inclusion in the diet, although this effect was not observed for n-3 LC-PUFA (*P*=0.669). Oil supplementation had no effect on Total n-6 PUFA (*P*=0.496) but decreased (*P*=0.001) the concentration of n-6 LC-PUFA in meat.

Table 2.4 - Effects of dietary *Cistus ladanifer* (Cistus) and oil supplementation (Oil) on intramuscular fat (IMF, g/100 g meat), and fatty acid concentration (mg/100 g meat) in lamb meat.

	Cistus ladanifer			**Oil**			**SEM**	***P* value**	
	5	**10**	**20**	**0**	**4**	**8**		**Cistus**	**Oil**
IMF	1.93	2.14	2.17	2.06	2.06	2.10	0.120	0.312	0.966
Total FA	1102	1271	1434	1348	1234	1224	134	0.253	0.774
FA composition									
SFA(12:0 to 16:0)	278	326	383	368	314	303	40.1	0.219	0.494
18:0	155	172	188	189	170	157	16.7	0.416	0.418
Total SFA	455	522	596	586	507	481	59.0	0.275	0.447
11*t*-18:1	17.7	19.3	26.7	18.3	21.7	23.6	2.55	0.058	0.355
Other *trans 18:1*	29.0^{a}	42.5^{b}	65.0^{b}	24.3^{a}	48.6^{b}	63.6^{b}	7.55	0.017	0.010
Total *trans*-MUFA	46.7^{a}	61.8^{a}	91.5^{b}	42.6^{a}	70.4^{ab}	87.0^{b}	9.19	0.014	0.016
9*c*-18:1	334	392	416	453	365	325	47.4	0.473	0.188
Other *cis*-MUFA	45.6	53.6	58.1	57.4	49.8	50.2	5.92	0.313	0.572
Total *cis*-MUFA	380	446	475	510	415	375	52.9	0.453	0.217
18:2 n-6	91	102	116	92	101	115	6.86	0.067	0.087
n-6 LC PUFA	30.1	28.1	25.8	35.4^{b}	25.2^{a}	23.4^{a}	1.89	0.324	0.001
Total n-6 PUFA	121	130	143	128	127	139	7.9	0.206	0.496
18:3 n-3	28.8	32.7	39.6	15.1^{a}	36.5^{b}	49.5^{c}	3.75	0.166	<0.001
n-3 LC PUFA	25.8	23.2	21.4	22.7	24.5	23.2	1.90	0.142	0.669
Total n-3 PUFA	54.6	55.9	61.0	37.8^{a}	60.9^{b}	72.7^{b}	4.91	0.634	<0.001
Total PUFA	176	186	204	166	188	212	12.6	0.329	0.066
CLA	8.1	8.6	11.3	9.4	8.9	9.7	1.42	0.248	0.913

IMF - intramuscular fat; FA - fatty acids; SFA- saturated fatty acids; *trans*-MUFA - *trans* monounsaturated fatty acids; *cis*-MUFA – *cis* monounsaturated fatty acids; PUFA - sum of n-6 + n-3 polyunsaturated fatty acids; n-6 PUFA - sum of n-6 PUFA (18:2 n-6 +18:3 n-6 + 20:2 n-6 + 20:4 n-6 + 22:4 n-6); n-3 PUFA - sum of n-3 PUFA (18:3 n-3 + 20:3 n-3 + 20:5 n-3 + 22:5 n-3 + 22:6 n-3); n-6 LC- PUFA - sum of long chain n-6 PUFA (20:2 n-6 + 20:4 n-6 + 22:4 n-6); *n*-3 LC-PUFA - sum of long chain n-3 PUFA (20:3 n-3 + 20:5 n-3 + 22:5 n-3 + 22:6 n-3; CLA – 9*c*,11*t*-18:2 + 7*t*,9*c*-18:2.

2.4. Discussion

2.4.1. Growth performance and carcass traits

Lambs revealed good growth performances, comparing to other results from previous trials with MB lambs. The observed ADG (269±68.8 g) was probably below breed growth potential (Santos-Silva *et al.*, 2002b), but close to the results obtained in other trials with lambs of the same breed, fed with similar diets (Santos-Silva & Vaz Portugal, 2001; Bessa *et al.*, 2005).

Cistus ladanifer can partly replace the forage component in lamb diets without compromising growth performance if diets are kept isoenergetic and isoproteic. In the present trial, *C. ladanifer* represented 40% of the forage fraction in the diets that included 200 g/kgDM and allowed an average growth rate of 251 g, a DM intake of 115 g/d/kg $wt^{0.75}$ and a feed conversion ratio of 5.87. Those values were similar to the correspondents that were obtained

with lower levels of CT inclusion in the diet and are in accordance to Jerónimo *et al.* (2010b), where *C. ladanifer* replaced 28% of dehydrated lucerne in lamb's diet, corresponding to a level of *C. ladanifer* inclusion of 250 g/kgDM. In the present trial, raising the levels of *C. ladanifer* in diet determined an increase of the concentration of CT from an average basal level of 2.7 g/kg DM to 15.6 g/kg DM. According to Min *et al.* (2003), the inclusion of CT in ruminant diets in levels between 20–45 g/kg DM may have beneficial effects on rumen metabolism and productivity. In this study, even the highest values are below that range, which may explain the absence of effect on growth performances. It must be emphasized that in spite of the low nutritional value of *C. ladanifer*, the diets used in the present trial were balanced for metabolizable energy and protein. As intake was not reduced by the increase of *C. ladanifer* proportion in the diets, the lack of significance in growth rate was expectable.

The variability of ADG between individuals was high, particularly in the groups fed with the higher level of *C. ladanifer* and supplemented oil. Globally, the variation coefficient for ADG was of 26.9%. However, the groups fed with 200 g/kgDM of Cistus, showed variation coefficients of ADG of 40.0% and 46.8%, when the levels of oil inclusion were 4% or 8%, respectively. Despite, the lack of significance for the effects of *C. ladanifer* on growth performance traits, these results suggest that the productive response of some of the lambs may have been impaired by the diets with higher levels of inclusion of CT when associated with oil supplementation. In fact, the lambs submitted to the higher levels of CT (20%Cistus) and oil supplementation (8%), showed levels of intake (102 g/kg$W^{0.75}$) and growth rate (185 g/day) lower than average, justifying that the interaction Cistus × Oil was close to significance (P= 0.089).

Oil supplementation had no effect on ADG but caused a reduction of DMI up to 9.6% (Table 2). The reduction of DMI and maintenance of productive performance after increasing energy density of diets by adding lipid supplements is well understood, and occurs when net energy intake is not reduced (Palmquist, 1994).

Carcass fat cover visual evaluation was improved by the dietary inclusion of *C. ladanifer*, although this result was not validated by tissue composition of chump and shoulder, probably, because the differences in fatness were not very expressive. In a previous trial, Jerónimo *et al.* (2010b) feeding *C. ladanifer* to MB lambs observed a significant increase of 22% in subcutaneous fat proportion and a reduction of 7.2% in muscle in the same carcass cuts. In the present trial, ether extract has increased with the level of *C. ladanifer* in the diets, but in smaller extent than in Jerónimo *et al.* (2010b), which can justify the difference in the results. Whitney *et al.* (2011), using red berry juniper, a bush species rich in condensed tannins, as roughage source for Rambouillet feedlot lambs, observed that the inclusion of this tanniferous bush had no effect on carcass characteristics, including carcass adiposity.

The use of lipids as a supplement increases the energy density of diets, which can justify the slight raise of carcass adiposity that was observed and that is in accordance to Santos-Silva *et al.* (2004) and Bessa *et al.* (2005). However, the results may be influenced by the nature and level of lipid supplementation, the breed and growth phase and basal diet, justifying the absence or low influence of oil on carcass composition reported by several authors (Radunz, *et al.*, 2009; Manso *et al.*, 2009; Ferreira *et al.*, 2014).

2.4.2. Meat quality parameters

Meat colour coordinates at day 0 of storage were not affected by the inclusion of *C. ladanifer* in lamb diets in accordance to Jerónimo *et al.* (2012). However, other authors observed that the inclusion of different tanniferous plant species in lamb diets, originates lighter meat (Priolo *et al.*, 1998; Priolo *et al.*, 2002b; Priolo *et al.*, 2005), as a consequence of a reduction in blood hemoglobin (Priolo *et al.*, 2000). Our results do not support that suggestion, even for the higher levels of condensed tannins that occurred in groups fed with 20% of Cistus in diet (14.3 – 16.5 g/kg DM).

In the same way, supplementation up to 8% oil of lamb diets had no effect on meat colour parameters at day 0 of storage, confirming the results of Santos-Silva *et al.* (2004), Bessa *et al.* (2005), Manso *et al.* (2009) and Jerónimo *et al.* (2012). In contrast, the results of Radunz *et al.* (2009) on lambs, showed that the supplementation with a vegetable oil blend, composed by soybean and linseed oil (2:1 respectively) resulted in lighter meat. However, these authors concluded that the differences in objective colour reported in the study were relatively small and may have little practical relevance for consumers.

During meat storage occur changes in mioglobin biochemistry (Lee, Kim, Liang & Song, 2003) with direct effect on colour parameters of meat, depreciating the quality perceived by the consumers that associate red colour with freshness (Mancini & Hunt, 2005). Our results show that the increase of *C. ladanifer* in lamb's diet was effective to reduce the meat colour changes during 7 days of refrigerated storage. However, the improvement of meat colour stability was more pronounced when Cistus increased from 5 to 10%. Higher levels of *C. ladanifer* in the diet (20%) had equal effectiveness to reduce the colour changes than 10% of Cistus. Priolo *et al.* (2000) and Luciano *et al.* (2009b) observed that the inclusion of carob pulp and quebracho in ruminant diets reduces the meat colour degradation during storage. However, Jerónimo *et al.*, (2012), referred that inclusion of 25% of *C. ladanifer* in lamb's diet had no effect on colour variation after 7 days of storage.

According to Buckley *et al.* (1995), oxidation of lipids is a major cause of deterioration in the quality of muscle foods and can directly affect many quality characteristics such as flavour,

colour, texture, nutritive value, and safety of the food. *Cistus ladanifer* was effective in reducing the lipid oxidation in meat from oil supplemented and unsuplemented lambs, but the effect was more pronounced between 5 to 10% than between 10 to 20%. Those results are in accordance to Jerónimo *et al.* (2012) that referred that the inclusion of 25% of *C. ladanifer* in lamb diets reduced the lipid oxidation of lamb meat after 7 days of storage.

In recent years, plants and plant extracts rich in polyphenolic compounds have been extensively studied as possible sources of natural antioxidants in animal nutrition, replacing their synthetic counterparts. *Cistus ladanifer* contains high levels of different polyphenolic compounds, as phenolic acids and several flavonoids other than CT (Barrajon-Catalan *et al.*, 2010), and *in vitro* antioxidant activity of *C. ladanifer* phenolic extracts was reported previously (Andrade *et al.*, 2009; Barrajón-Catalán *et al.*, 2010). Therefore, the improvement of colour and lipid oxidative stability induced by *C. ladanifer* might result of the action of some polyphenolic compounds presents in *C. ladanifer*. However, there is no conclusive information about the nature of the bioactive compounds present in *C. ladanifer* extracts that are directly associated to the antioxidant capacity, as well as the action mechanisms underlying of such effect.

The colour and lipid oxidation of meat was not affected by dietary oil supplementation. The diets were supplemented with increasing levels of oil blend (from 0 to 8% of oil) rich in PUFA. The PUFA are known by high susceptibility for oxidation. However, oil supplementation of diets did not reflect in a significant increased of the PUFA content in meat, which may explain the absence of the effect of dietary oil supplementation on oxidative stability of meat.

Shear force values obtained in this trial were low (average 3.46±1.343 kg), indicating high tenderness of meat, and were not influenced by treatments. The values were similar to others reported to MB lambs, raised in similar conditions (Santos-Silva *et al.*, 2002b, 2004 and Bessa *et al.*, 2005). Previous studies concluded that the inclusion of tanniferous plants in ruminant diets did not affect meat tenderness evaluated by a taste panel (Priolo *et al.*, 1998; Whitney *et al.*, 2011) or by instrumental devices (Simitzis *et al.* 2008).

The panel results about the effects of *C. ladanifer* inclusion in the diet on lamb meat flavour and off-flavours are not clear, but may suggest that it can be associated to an increase of both parameters. However, the off-flavours were referenced only in 30% of the samples and in low levels. In addition, taking into account that overall acceptability scores were close to 5, it is not expected that they have a relevant role in consumer's acceptance or commercial value of meat. It is reported that inclusion of alternative vegetable feed sources, rich in secondary compounds, as tannins, can increase the off-flavours in lamb meat, as it was observed when diets were supplemented with redberry juniper (Whitney *et al.*, 2011). However, this effect might be highly dependent of the type and level of the feed source used.

Priolo *et al.* (1998) reported that the inclusion of carob pulp in lamb's diet could not be detected when meat was experienced by a trained sensory panel. Our research team reported that 25% inclusion of *C. ladanifer* in lamb diets had no effect on meat sensory properties assessed by consumers (Jerónimo *et al.*, 2012) even if the inclusion of *C. ladanifer* in lamb diets could be chemically traced by the presence of distinct volatiles profile released by meat heated at 60 °C (Vasta *et al.*, 2010b). Notably, that in meat from lambs fed with *C. ladanifer* was found 2,2,6-trimetyl-ciclohexanone and verbenone, which were transferred directly from *C. ladanifer* to lamb meat (Vasta *et al.*, 2010b).

Supplementation with 8% oil increased sensory panel scores for off-flavour intensity and simultaneously the level of *n*-3 PUFA in meat (Table 2.3), that are particularly prone to oxidative degradation and to the production of volatiles that are known to be associated to the development of off-flavours in meat (Nute *et al.*, 2007). Therefore, the increment of meat off-flavour intensity with oil supplementation is probably related to the higher *n*-3 FA content of meat, as suggested by the positive correlation between those two variables (r=0.34; *P*=0.012, data not shown). In general, oil supplementation slightly decrease the overall acceptability of meat by consumers, contrarily to what it was observed in previous studies of our research group, where it was concluded that lipid supplements had minor sensory effects (Santos-Silva *et al.*,2003; Santos-Silva *et al.*, 2004; Bessa *et al.*, 2005).

2.4.3. Meat nutritional value

The values of total fat content of muscle obtained in this study (averaging 2% in fresh meat) were below the 5% IMF, which is the reference value to consider meat as a low fat food (FAC, 1990). Furthermore, the values obtained for IMF were close to others obtained in the same breed in similar production systems (Santos-Silva *et al.*, 2004; Bessa *et al.*, 2005; Jerónimo *et al.*, 2010b). Detailed FA composition of these lamb meat samples will be published elsewhere, but here we report the concentration of partial sums and of few individual FA with nutritional relevance. The SFA and *cis*-MUFA comprised the majority of the FA, as expected in lamb meat, and were fairly unaffected by treatments. We had previously observed that 25% of dietary inclusion of *C. ladanifer* did not significantly affect the sum of SFA (Jerónimo *et al.*, 2012), but supplementation with unsaturated oils commonly reduces the sum of SFA, and 9*c*-18:1 (Jerónimo *et al.*, 2012). In fact, when expressed in percentage of total meat FA (data not shown), we observed a significant decrease of SFA, including 18:0, and also of the 9*c*-18:1, but when expressed in mg/100 g of fresh meat, no significant differences were detected. The recent nutritional recommendations indicate that SFA should not represent more than 10% of energy intake (FAO, 2010), which for a normal range of daily energy intake for adults (2 to 3.5 Mcal) will correspond to 24 to 36 g of SFA.

Although rich in SFA, the lean lamb meat may not be considered a major contributor to total SFA intake, as 100 g of meat will supply only 0.5 g.

Intake of *trans* FA have been convincingly related to deleterious health effects and its intake is recommended to be lower than 1% of energy daily intake, which correspond to 2.4 to 3.9 g for the energy intakes that was referred above (2 to 3.5 Mcal) (FAO, 2010). In this trial concentration of *trans* FA was below 100 mg/100 g of fresh meat. Most of studies on *trans* FA health effects are focused on those derived from partially hydrogenated vegetable oil, extensively used in the past by the food industry. However, in our days the ruminant edible fats are gaining relative importance as dietary sources of *trans* FA (Aldai *et al.*, 2013). The profile of individual *trans* FA of ruminant fats is normally dominated by the 18:1 *trans*-11, that is the major precursor for endogenously CLA synthesis (mostly 18:2 *cis*-9,*trans*-11 isomer) and a common target for the improvement of ruminant nutritional value of fat (Mapiye *et al.*, 2012). Both *C. ladanifer* and oil increased the concentration in meat of total *trans* 18:1 FA, but not the 11*t*-18:1 neither CLA. In a previous experiment we found that dietary Cistus increased both 11*t*-18:1 and CLA in diets supplemented with oil (Jerónimo *et al.*, 2012). The reasons for such a difference are probably associated to the composition of basal diet that was dehydrated lucerne in the previous trial and a compound feed that included cereals in the present trial.

The oil blend used as a supplement in this trial was composed by two parts of linseed oil and one part of soybean oil. As linseed is rich in 18:3 n-3, the concentration of this fatty acid in meat increased. However, the concentration of the n-3 LC-PUFA was unaffected by oil supplementation. The absence of increase of the n-3 LC-PUFA after supplementation with linseed oil have been previously observed (Bessa *et al.*, 2007; Albertí *et al.*, 2014) and can be explained by the fact that increased availability of 18:3 n-3 inhibits its metabolization into EPA, DPA and DHA, as it was reported long ago by Holman (1986). The average amount of EPA plus DHA was 11.71 mg/100 g of lamb meat, which corresponds to 4.7% of the recommended daily intake for adults by European Food Safety Authority [EFSA] (2010).

2.5. Conclusions

In isoenergetic and isonitrogenous diets, incorporating 10% of *C. ladanifer* in the diet of lambs seems to be a good compromise, given the set of variables that were studied. It allowed good growth performances, had no effects on carcass composition, sensory traits and nutritional value of meat, and improved the oxidative stability of colour and lipids after 7 days of refrigerated storage. The level of 20% of *C. ladanifer* in lamb's diet, when associated

to lipid supplementation, is probably close to the physiologic tolerance of rumen ecosystem, and was associated to a high variability in individual growth performance traits.

The level of oil supplementation of 8% DM, is probably close to the upper limit that does not compromise lambs productivity and meat quality. Chemical and physical meat quality traits were not affected, but sensorial evaluation has been negatively influenced in some traits and overall acceptability decreased slightly. The use of a blend of linseed and soybean oils (2:1) was a good approach to improve meat nutritional value of meat from feedlot lambs, maintaining a low fat content, but promoting the level of Total n-3 fatty acids up to 72.7 mg/100 g of meat.

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CHAPTER 3

Effect of feeding lambs with a tanniferous shrub (rockrose) and a vegetable oil blend on fatty acid composition of meat lipids

Effect of feeding lambs with a tanniferous shrub (rockrose) and a vegetable oil blend on fatty acid composition of meat lipids

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Contribution of Alexandra E. Francisco to this article:

Alexandra E. Francisco participated in the peleting of the diets, in the animal experiment and in the sample collection. Determined the fatty acid content and performed the data processing. Furthermore, Alexandra E. Francisco participated in the statistical analysis, interpretation and discussion of the results, as well as in the writing of the manuscript.

ABSTRACT

The effects of feeding *Cistus ladanifer* and a blend of soybean and linseed oil (1:2 vol/vol) on fatty acid (FA) composition of lamb meat lipids and mRNA expression of desaturase enzymes was assessed. Fifty four male lambs were randomly assigned to 18 pens and to 9 diets, resulting from the combination of 3 inclusion levels of *C. ladanifer* (50 *vs* 100 *vs* 200g/kg of dry matter (DM)) and 3 inclusion levels of oil (0 *vs* 40 *vs* 80g/kg of DM). The forage-to-concentrate ratio of the diets was 1:1. *Longissimus* muscle lipids were extracted, fractionated into neutral (NL) and polar lipid (PL) and FAME obtained and analyzed by GLC. The expression of genes encoding Δ5, Δ6 and Δ9 desaturases (*FADS1*, *FADS2* and *SCD*) was determined. Intramuscular fat, NL and PL contents were not affected by oil or *C. ladanifer*. Oil supplementation reduced ($P < 0.05$) 16:0, 9*c*-16:1, 17:0, 9*c*-17:1 and 9*c*-18:1 FA and increased ($P < 0.05$) 18:2 n-6, 18:3 n-3 and the majority of BI in NL. *Cistus ladanifer* alone had few effects on FA of NL but interacted with oil ($P < 0.05$) by increasing 10*t*-18:1, 10*t*,12*t*-18:2, 10*t*,12*c*-18:2 and 7*t*,9*c*-18:2. The 10*t*-/11*t*-18:1 ratio increased with both Cistus and oil levels. The 9*c*,11*t*-18:2 did not increase ($P < 0.05$) with both oil and *C. ladanifer* dietary inclusion. Oil reduced 9*c*-16:1, 17:0, 9*c*-17:1, 9*c*-18:1, 20:4 n-6, 22:4 n-6 and 20:3 n-9 proportions in PL, and increased 18:2 n-6, 18:3 n-3, 20:3 n-3 and of most of the BI. The Cistus had only minor effects on FA composition of PL. *Cistus ladanifer* resulted in a reduction ($P < 0.05$) of 20:5 n-3 and 22:6 n-3 in the meat PL. The expression level of *SCD* mRNA was increased ($P = 0.015$) with Cistus level, although a linear relationship with condensed tannins intake ($P = 0.11$) could not be established. *FADS1* mRNA expressed levels increased linearly ($P = 0.019$) with condensed tannins intake. In summary, the inclusion of *C. ladanifer* and oil in 1:1 forage-to-concentrate ratio diets resulted in a large increase in 10*t*-18:1 and no increase in 9*c*,11*t*-18:2 or n-3 LC-PUFA in lamb meat.

Key words: Condensed tannins; Lipid supplementation; Biohydrogenation intermediates; *trans*-10 shift; Fatty acid desaturases

Implications

Cistus ladanifer (rockrose) is a tanniferous shrub widespread in Mediterranean marginal lands. Tannins can modify the metabolism of unsaturated fatty acids in the rumen and increase the health promoting fatty acids in meat or milk. When rockrose was incorporated in oil supplemented finishing diets for lambs, it modified the biohydrogenation activity in the rumen, promoting a large accumulation of 10*t*-18:1 in meat lipids. The 10*t*-18:1 is not a desirable fatty acid in meat, hence some caution is needed when tanniferous plants are used as diet ingredient to improve the fatty acid profile of lamb meat.

3.1. Introduction

Ruminant edible fats are rich in SFA, poor in PUFA, and have a variable content of TFA. Therefore, they are generally regarded as unhealthier attending to the current nutritional guidelines for human health (FAO, 2010). Nevertheless, ruminant edible fats are the richest dietary source of CLA, particularly of 9*c*,11*t*-18:2, which has been shown to possess anticancerogenic effects in several animal models (Shingfield & Wallace, 2014). Thus, nutritional strategies to increase CLA and PUFA content in ruminant edible fat have been intensively investigated in the last 2 decades.

Increasing PUFA intake results in larger rumen outflow of PUFA and biohydrogenation intermediates, thus promoting increased depositions of PUFA and CLA in tissues (Shingfield *et al.*, 2013). However, due to the extensive diversity of rumen microbial isomerization and hydrogenation metabolism (Biohydrogenation), the increase in PUFA and CLA in meat can be quite variable, stressing the need to find biohydrogenation modulators.

Tannins and tannins sources have been proposed as potential modulators of biohydrogenation, being able to disturb the last reductive step of biohydrogenation thus increasing the availability of 11*t*-18:1 that will support the synthesis of 9*c*,11*t*-18:2 through the action of Δ9-desaturase in tissues (Vasta & Bessa, 2012). Moreover, it has been suggested that dietary tannins might up-regulate *SCD* expression levels (Vasta *et al.*, 2009c; Rana *et al.*, 2012).

Cistus ladanifer is a tanniferous shrub, occurring abundantly in Mediterranean marginal lands (Guerreiro *et al.*, 2015). Jerónimo *et al.* (2010b) reported a significant increase of 11*t*-18:1 and 9*c*,11*t*-18:2 in lamb meat lipids with the addition of *C. ladanifer* to an oil supplemented diet. Although promising, these results were obtained with high dietary incorporation of *C. ladanifer* (25% DM) and vegetable oil (6% DM) into a dehydrated alfalfa basal diet. However, the effect of these dietary supplements on conventional diets (with higher starch content) for growing ruminants remains to be established.

High starch diets generally stimulate adipogenesis and lipogenesis including Δ9-desaturase activity, which favors CLA synthesis and tissue deposition. However, these types of diets promote distinct biohydrogenation pathways compared with those found in forage-based diets, with accumulation of 10*t*-18:1 instead of 11*t*-18:1. This results in decreased availability of 11*t*-18:1 as substrate for Δ9-desaturase (Bessa *et al.*, 2015). We hypothesize that a basal diet with a forage-to-concentrate ratio of 1:1 would promote both a high availability of 11*t*-18:1 and an up-regulation of Δ9-desaturase and in that conditions a lower addition of *C. ladanifer* and oil would be needed to achieve high CLA content in lamb meat. Therefore, the

general aim of the present study was to elucidate if the combined effects of *C. ladanifer* and oil on lamb meat FA composition reported by Jerónimo *et al.* (2010b) could be achieved using a basal diet with 1:1 forage-to-concentrate ratio, and with lower dietary incorporation of *C. ladanifer* or vegetable oil.

3.2. Material and methods

3.2.1. Experimental design and animal management

All the experimental procedures involving animals were approved by the Animal Care Committee of the National Veterinary Authority (Direção Geral de Alimentação e Veterinária, Lisbon, Portugal), following compliance guidelines of European Union (Directive 86/609/EEC).

Detailed information about diets, animal handling, slaughter, sample collection and analytical procedures has been reported by Francisco *et al.* (2015). Briefly, 54 Merino Branco ram lambs, weighing 16.2 ± 2.93 kg, ageing 78 ± 7.7 d (mean ± SD), were randomly assigned to 18 pens of 3 lambs each, and 2 pens per treatment, according to a completely randomized experimental design with a 3×3 factorial arrangement of treatments. The first factor was the effect of 3 levels (50 vs 100 vs 200 g/kg of dry matter (DM)) of incorporation of dried and grounded *C. ladanifer* leaves and soft stems in diets composed by a mixture of dehydrated lucerne, wheat, maize and soybean meal. The second factor was the effect of 3 levels (0 vs 40 vs 80 g/kg of DM) of oil supplementation. The 9 diets resulting from the 3×3 factorial arrangement were: 1) 5CL (50 g Cistus/kg DM); 2) 10CL (100 g Cistus/kg DM); 3) 20CL (200 g Cistus/kg DM); 4) 5CL4O (50 g Cistus/kg DM + 40 g oil/kg DM); 5) 10CL4O (100 g Cistus /kg DM + 40 g oil/kg DM); 6) 20CL4O (200 g Cistus /kg DM + 40 g oil/kg DM); 7) 5CL8O (50 g Cistus/kg DM + 80 g oil/kg DM); 8) 10CL8O (100 g Cistus/kg DM + 80 g oil/kg DM) and 9) 20CL8O (200 g Cistus/kg DM + 80 g oil/kg DM). The oil used was a blend of soybean (*Glycine max*) and linseed (*Linum usitatissimum*) oils (1:2 vol/vol). The FA composition of the 9 diets is presented in Table 3.1. The formulas of the diets and their detailed chemical composition, including the content in total phenols and condensed tannins, are reported in Francisco *et al.* (2015).

Table 3.1- Total phenols, condensed tannins and fatty acids content and composition (g/ kg DM) of diets resulting from the combination of the incorporation of 3 levels of vegetable oil (O) and 3 levels of *Cistus ladanifer* (CL)

	0% Oil			4% Oil			8% Oil[1]		
	5% CL	**10% CL**	**20% CL**	**5% CL**	**10% CL**	**20% CL**	**5% CL**	**10% CL**	**20% CL**
Total phenols	5.9	10.5	20.0	5.8	10.0	21.5	7.6	12.6	22.2
Condensed tannins	2.5	6.5	16.3	2.9	6.9	14.5	2.6	7.4	16.1
Total fatty acids	12.4	16.0	16.3	37.7	42.6	45.3	44.0	71.8	62.1
Fatty acids									
16:0	3.01	3.11	3.00	5.38	5.47	5.00	5.40	7.66	6.95
18:0	0.49	0.69	0.86	1.76	1.91	2.03	2.06	2.93	3.10
9*c*-18:1	2.30	3.83	3.60	8.44	11.06	11.32	9.64	17.6	13.9
18:2 n-6	5.74	7.52	7.82	13.0	14.6	15.7	15.4	23.0	19.3
18:3 n-3	0.92	0.88	1.04	9.11	9.55	11.29	9.50	20.6	19.0

[1] Soybean and linseed oil blend (1:2 vol/vol)

3.2.2. Slaughter and sample collection

The experimental trial lasted for 6 weeks, and after that time lambs were stunned and slaughtered by exsanguination in the experimental abattoir of the INIAV. Average slaughter weigh was 32.5 ± 4.14 kg and it was not affected by treatments. For gene expression analysis, samples of *Longissimus* muscle were collected immediately after slaughter at the level of 12th vertebra, flash-frozen in liquid nitrogen and preserved at -80ºC until analysis. Carcasses were chilled at 2°C until the third day after slaughter, when meat samples were collected. For the determination of intramuscular fat and fatty acid composition, *longissimus* muscle was isolated from the rib joint. The *epimysium* was removed and muscle samples were minced using a food processor (3×5s), vacuum-packed, freeze-dried and stored at -20°C until analyses.

3.2.3. Analytical procedures

3.2.3.1. Intramuscular lipid composition

Fatty acid methyl esters of feed lipids were obtained by a one-step extraction transesterification, with toluene and heptadecanoic acid (17:0) as internal standard, according to Sukhija and Palmquist (1988).

Intramuscular lipids were extracted using a mixture of dichloromethane and methanol as described in Bessa *et al.* (2007). Polar (PL) and neutral (NL) lipid fractions were obtained by solid-phase extraction using dichloromethane and methanol and silica gel cartridges (Sep-Pack Chromabond® SiOH, 3 mL / 500 mg). For the separation of NL fraction the total lipids were eluted with 30 mL of dichloromethane. The PL were obtained by sequential elution with 30 mL of methanol. In both fractions, FA were transesterified with sodium methoxide (0.5 N) in methanol during 30 minutes at 50ºC, followed by hydrochloric acid in methanol (1:1 v/v) during 10 minutes at 50ºC. For analysis of the FA composition, FAMEs were analyzed using a HP6890A gas chromatograph (Agilent, Avondale, PA, USA), equipped with a flame-ionization detector and a SP-2560 fused silica capillary column (100 m, 0.2 mm i.d., 0.20 µm film thickness; Supelco, Bellefonte, PA, USA). The injector and detector temperatures were 250 and 280°C, respectively. Initial oven temperature of 100°C was held for 1 min, increased at 50°C/min to 150°C and held for 20 min, increased at 1°C/min to 190°C and held for 5 min, and then increased at 1°C/min to 200°C and held for 35 min. Helium was used as carrier gas at a flow rate of 1 mL/min, the split ratio was 1:30 and 1 µL of sample was injected. Nonadecanoic acid (19:0) was used as internal standard for FAMEquantification. Identification of FAMEwas achieved by comparison of the FAMEretention times with those of

commercial standard mixtures (FAME mix 37 components from Supelco Inc., Bellefont, PA, USA) and with published chromatograms (Alves & Bessa, 2009 and 2014). Additional identification of the FAME was achieved by electron impact mass spectrometry using a Shimadzu GC-MS QP2010 Plus (Shimadzu, Kyoto, Japan).

The methyl esters of CLA isomers were individually separated by triple silver-ion columns in series, using a high performance liquid chromatography system equipped with auto-sampler and diode array detector (DAD) adjusted to 233 nm. Regarding the quantification of the individual CLA isomers in meat, a combination of gas chromatography and 3 Ag^{+}-HPLC was used, as described in Bessa *et al.* (2007).

3.2.3.2. RNA extraction and qPCR assays

Total RNA was isolated from lamb muscle samples by using QIazol reagent and further purified using the RNeasy mini kit with on column DNase I treatment based on the manufacturer's protocol (all from Qiagen, Hilden, Germany). RNA yield was measured with the NanoDrop® ND-2000c spectrophotometer (Thermo Scientific, Wilmigton, DE, USA) and only A260/280 ratios between 1.9 and 2.1 were considered acceptable.

For each sample, 0.5 µg RNA was reversely transcribed using random primers with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. For the negative controls, the use of the enzyme reverse transcriptase (RT negative control) was omitted. PCR primers were designed based on mRNA sequences to cross at least one exon/exon junction (GenBank accession number XM_004019593 for FADS1, XM_004019592 for FADS2, NM_001009254 for SCD, NM_001009784 for ACTB and XM_004017413 for RPLP0) using Primer3 software (Untergasser *et al.*, 2012) and Primer Express software v3.1 (Applied Biosystems, Foster City, CA, USA), and produced amplicons of 76 to 146 bp size. All primers were purchased from NZYTech, Lisbon, Portugal. The nucleotide sequences of the primers used for qPCR studies and sizes of the PCR products are shown in Table 3.2. The stability of eight potential references genes (B2M, ACTB, GAPDH, RPLP0, Cyclophilin B, 18s, SDHA, RPS9) (primer pairs used are available on request) was evaluated using geNorm and NormFinder, as described by Vandesompele *et al.* (2002) and Andersen, Jensen and Orntoft (2004), respectively, whereas ACTB and RPLP0 was the suitable pair of reference genes. All primer pairs were validated by designing relative standard curves for gene transcripts with serial 5-fold dilutions of the pooled cDNA sample. The efficiency of each primer pair was calculated according to the standard curve method using the equation E = 10-1/slope, and was found within the range of 90%–110%. Quantitative PCR was performed to study expression of sheep *FADS1*, *FADS2* and *SCD* mRNAs by using the Power SYBR® Green master mix

(both Applied Biosystems, Foster City, CA, USA) on a StepOnePlusthermocycler at standard cycling conditions (Applied Biosystems, Foster City, CA, USA). No transcription and no template samples were used as controls. Melt curve analysis and the amplification efficiency were used to verify the specificity of the amplification, followed by gel electrophoresis. All analyses were performed in duplicate, and the relative amounts for each target gene were normalized using the geometric mean of references genes. Relative expression levels were calculated as a variation of the Livak method (Livak & Schmittgen, 2001), corrected for variation in amplification efficiency.

Table 3.2- Specifications of oligonucleotides used for RT-qPCR

Gene Symbol	Full gene name	Primerpairs (5′-3′)	Ampliconlength (bp)
Rplp0	Ribosomal protein, large, P0	F – ACTCTGCATTCCCGCTTCCT R – ACGCTTGTACCC ATTGATGA	114
ACTB	actin, beta	F – GGCCAACCGTGAGAAGATGA R – GCGTACAGGGACAGCACAGC	93
FADS1	Fatty_acid_desaturase 1	F - TTGGGGAAGGTCCTGTCTGT R – GGAAGTAGACAGGCAGCAAGG	118
FADS2	Fatty_acid_desaturase 2	F – CTTCCACCGCAACCTTGATT R – TGTCCTCAGCAGTCTTCCTCAG	146
SCD	stearoyl-CoA desaturase	F – CCATCAACCCCCGAGAGAAT R – AAGGTGTGGTGGTAGTTGTGGAA	76

F = forward primer; R = reverse primer

3.2.4. Statistical analysis

Data were analysed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) being *C. ladanifer* inclusion, oil supplementation and their interaction included in the model as fixed effects. The pen was the experimental unit and lambs were considered as repeated measures within each pen, using a compound symmetry covariance matrix. The variance homogeneity was checked and when justified the variance heterogeneity structure was accommodated in the model. The $P < 0.05$ was set as the level of statistical significance.
It was observed a significant interaction between *C. ladanifer* and oil levels for the intake of C18 unsaturated FA (Table 3.3) that could mask the effects of *C. ladanifer* on muscle FA composition. To overcome this constraint, the FA that were significantly affected by *C. ladanifer* or Cistus×Oil interaction, were submitted to a complementary regression analysis with the Proc GLM of SAS, where the effects of average daily intake of C18 unsaturated FA and of condensed tannins in each pen were tested as continuous independent variables and the average meat FA composition in each pen as dependent variables. Pearson correlations among variables were conducted when necessary.

3.3. Results

Data concerning productive performance of lambs, carcass traits and meat quality results have been reported elsewhere (Francisco *et al.*, 2015). Here we describe the detailed fatty acid composition of lamb meat lipids and the expression of level of desaturase genes.

3.3.1. Intake of fatty acid and condensed tannins

An interaction between the levels of oil and CT in the diets was observed for total FA intake and for individual FA intake, except for 16:0 and 18:0 (Table 3.3). For diets with 0% and 4% oil, increasing levels of *C. ladanifer* increased the intake of 9*c*-18:1, 18:2 n-6 and 18:3 n-3, which was more expressive for diets with 4% oil. However, for 8% oil, FA intake only increased when *C. ladanifer* increased from 5 to 10%, decreasing thereafter.
The intake of total phenols and CT reflected directly the level of *C. ladanifer* inclusion in the diets and CT rose from an average of 3.7 g/d, for 5% of Cistus up to 21.0 g/d for 20% of Cistus.

Table 3.3- Effects of *Cistus ladanifer* (CL) and vegetable oil (Oil) on dry matter, and fatty acid, total phenols and condensed tannins intake (g/day) of lambs.

	0% Oil			4% Oil			8% Oil[1]				*P* values		
	5%CL	10%CL	20%CL	5%CL	10%CL	20%CL	5%CL	10%CL	20%CL	SEM	Oil	CL	Oil×CL
Dry matter	1341	1305	1438	1275	1416	1504	1318	1417	1153	93.7	0.42	0.65	0.18
Fatty acids													
16:0	4.08	4.07	4.33	6.84	7.73	7.53	7.10	10.77	7.97	0.571	<0.001	0.024	0.053
18:0	0.68	0.92	1.24	2.23	2.69	3.05	2.70	4.11	3.54	0.229	<0.001	0.006	0.17
9*c*-18:1	3.23^{d}	5.05^{d}	5.24^{d}	10.8^{c}	15.6^{b}	17.0^{b}	12.7^{bc}	24.7^{a}	15.9^{b}	1.391	<0.001	0.001	0.016
18:2 n-6	7.88^{e}	9.87^{e}	11.3^{de}	16.6^{cd}	20.6^{bc}	23.5^{b}	20.2^{bc}	32.3^{a}	22.1^{b}	1.739	<0.001	0.005	0.020
18:3 n-3	1.40^{d}	1.29^{d}	1.63^{d}	11.6^{c}	13.5^{c}	16.9^{bc}	12.5^{c}	28.9^{a}	21.6^{b}	1.716	<0.001	0.005	0.007
Total fatty acids	17.3^{d}	21.2^{d}	23.7^{d}	48.0^{c}	60.1^{bc}	68.1^{b}	55.2^{bc}	101^{a}	71.1^{b}	5.58	<0.001	0.004	0.019
Total phenolics	8.11	13.8	28.6	7.58	14.27	32.2	10.2	17.9	25.3	1.77	0.68	<0.001	0.07
Condensed tannins	3.56	8.57	23.25	3.89	9.87	21.8	3.62	10.6	18.0	1.36	0.54	<0.001	0.17

[1] Soybean and linseed oil blend (1:2 vol/vol)

[a,b,c,d] Values within a row with different superscripts differ significantly at $P<0.05$

3.3.2. Muscle lipids

Total muscle lipids were extracted and fractionated into PL and NL and their FA composition was determined and is presented in Tables 3.4 to 3.7. The gravimetric contents, of total lipids and NL (Table 3.4), and PL (Table 3.6) was not affected by treatments and averaged 37.3, 20.1 and, 13.0 mg/g of fresh meat, respectively. The NL and PL accounted for 89% of total lipids mass. Consistently, the FA content in NL, PL and total lipids, calculated from GLC internal standard method, was also not affected by treatments and averaged 12.1, 3.04, and 20.8 mg/g of fresh muscle, respectively. The sum of FA content of NL and PL fractions accounted for 73.3% of FA content of total lipids.

3.3.2.1. Fatty acid composition of muscle neutral lipids

The FA composition of NL is presented in Tables 3.4 and 3.5. The major FA in NL was 9*c*-18:1, followed by 16:0 and 18:0. The proportion of 16:0, 9*c*-16:1, 17:0, 9*c*-17:1 and 9*c*-18:1 was reduced by oil supplementation whereas 18:2 n-6 and 18:3 n-3 increased 43.6% and 242%, respectively. Also, the proportions of majority of the BI, including 11*t*-18:1, increased with oil supplementation, resulting in a higher sums of 18:1 isomers, and 18:2 non-conjugated and conjugated isomers.

Table 3.4 -Effects of *Cistus ladanifer* (CL) and vegetable oil (O) on total lipids, neutral lipid fraction (NL), total fatty acids (FA) in NL (mg/g muscle) and fatty acid profile of NL (mg /g of total NL FA) of longissimus muscle of lambs.

	0% Oil			4% Oil			8% Oil[1]			SEM	*P* values		
	5%CL	10%CL	20%CL	5%CL	10%CL	20%CL	5%CL	10%CL	20%CL		O	CL	O×CL
Total lipids	36.7	36.8	38.1	32.8	40.7	37.2	35.0	37.3	42.2	3.54	0.90	0.32	0.70
LN	18.4	19.8	21.7	17.3	23.6	20.6	18.2	18.8	23.9	3.24	0.98	0.34	0.79
Total FA in LN	11.3	12.9	13.5	9.2	15.3	12.5	9.5	12.5	14.0	2.50	0.86	0.25	0.77
FA profile													
14:0	27.2	35.0	46.5	41.6	30.8	35.7	37.2	33.3	35.3	3.29	0.96	0.23	0.36
i-15:0	1.1	1.0	1.1	1.3	0.9	1.0	1.2	1.0	1.0	0.15	0.94	0.18	0.91
a-15:0	1.7	1.4	1.6	2.0	1.3	1.3	1.5	1.6	1.5	0.29	0.97	0.39	0.67
9*c*-14:1	1.2	1.2	1.8	1.4	1.0	1.2	1.3	1.1	1.2	0.17	0.40	0.21	0.23
15:0	4.6	4.2	4.9	5.2	4.0	3.7	3.9	4.0	4.2	0.41	0.36	0.34	0.24
i-16:0	1.3	1.2	1.4	1.5	1.0	1.1	1.3	1.1	1.3	0.16	0.81	0.16	0.63
16:0	252^{ab}	250^{b}	286^{a}	236^{b}	246^{b}	251^{b}	238^{b}	240^{b}	235^{b}	6.6	0.004	0.06	0.086
i-17:0	3.1	2.8	3.2	3.8	2.7	2.8	2.9	3.0	3.0	0.47	0.95	0.50	0.69
7*c*-16:1	3.5	3.2	3.1	3.8	2.9	3.1	3.2	3.4	3.2	0.33	0.99	0.36	0.54
9*c*-16:1	19.0	19.3	20.9	18.6	16.8	15.9	16.6	16.8	16.0	1.10	0.012	0.86	0.36
17:0	15.1	13.5	12.3	13.5	11.9	10.0	11.2	10.7	11.6	0.68	0.005	0.018	0.14
i-18:0	1.6	1.1	1.2	1.5	1.0	1.1	1.2	1.1	1.2	0.12	0.29	0.020	0.51
9*c*-17:1	6.3	6.4	5.1	5.5	4.9	3.9	4.5	4.4	4.8	0.48	0.014	0.13	0.33
18:0	166	154	140	151	147	140	146	136	135	6.3	0.061	0.041	0.75
9*c*-18:1	372	381	333	335	349	300	316	305	292	13.9	0.003	0.023	0.69
11*c*-18:1	11.9	11.1	10.7	11.2	11.0	9.0	10.2	10.2	9.8	0.53	0.063	0.048	0.38
18:2 n-6	24.6	24.7	26.6	29.1	30.2	37.2	33.1	36.6	38.7	1.82	<0.001	0.018	0.44
18:3 n-3	7.6	7.2	8.5	16.3	17.0	23.0	24.0	26.9	27.8	1.11	<0.001	0.007	0.10
20:0	1.3	1.3	2.3	1.5	1.6	2.1	1.6	1.2	1.7	0.17	0.34	0.002	0.21
11*c*-20:1	0.8	0.8	0.8	0.8	0.8	1.0	0.9	0.8	1.0	0.07	0.23	0.11	0.81
20:2 n-6	0.6	0.6	0.6	0.8	0.8	0.8	0.7	0.8	0.8	0.12	0.15	0.96	0.92
22:0	0.6	0.4	0.3	0.5	0.3	0.4	0.1	0.2	0.2	0.10	0.028	0.55	0.37

20:4 n-6	2.4	2.0	1.7	2.5	1.5	1.6	1.7	1.5	1.5	0.31	0.19	0.09	0.74
20:5 n-3	1.0	0.7	1.0	1.3	0.8	0.8	1.1	0.9	0.9	0.18	0.92	0.18	0.60
22:5 n-3	1.6	1.2	1.4	2.2	1.3	1.4	1.5	1.3	1.4	2.64	0.44	0.11	0.67

[1] Soybean and linseed oil blend (1:2 vol/vol)

[a,b,c,d] Values within a row with different superscripts differ significantly at $P < 0.05$

Cistus ladanifer inclusion reduced the proportions of 17:0, iso-18:0, 18:0, 9*c*-18:1 and 11*c*-18:1 and increased the 18:2 n-6, 18:3 n-3, 20:0. In addition Cistus increased total BI, mainly due to its effects on increasing 9*t*-18:1, 10*t*-18:1 and on the unresolved peaks of 6*t*-/7*t*-/8*t*-18:1 and of 10*t*,15*c*-/11*t*,15*c*-18:2.

Significant interactions between *C. ladanifer* and oil supplementation were observed for 10*t*-18:1, 10*t*,12*t*-18:2, 10*t*,12*c*-18:2 and 7*t*,9*c*-18:2 (Table 3.5). For these FA it was observed a synergic effect between *C. ladanifer* and oil supplementation, that was particularly expressive for 10*t*-18:1 and 10*t*,12*c*-18:2. For 10*t*-18:1 and 10*t*,12*t*-18:2 a great individual variability was observed when animal were fed with the diet containing the highest level of *C. ladanifer* and oil.

The 10*t*-18:1/11*t*-18:1 ratio (10*t*-/11*t*-) increased with both Cistus and oil levels. The only treatments that presented a 10*t*-/11*t*- below 1 were those non-supplemented with oil (5, 10 and 20 % of Cistus) and that with lowest Cistus level and 4% of oil (5CL4O).

The averages for selected individual FA obtained from each pen were submitted to complementary regression analysis using the average daily intake of C18 unsaturated FA and of CT as independent variables (Table 3.9). The intake of CT, adjusted for the same C18 unsaturated FA, had no effect on the 18:2 n-6, 18:3 n-3 and 11*c*-18:1 (Table 3.9), suggesting the effect observed for dietary *C. ladanifer* levels was due to the confounded effect of C18 unsaturated FA intake. Increasing CT intake caused an increase of 20:0, 6*t*-/7*t*-/8*t*-18:1, 9*t*-18:1 and a decrease with of 17:0, 18:0, 9*c*-18:1, even after adjustment for C18 unsaturated FA intake (Table 3.9). Moreover a significant interaction between FA and CT intake was observed for 10*t*-18:1, 10*t*,12*t*-18:2 and 10*t*/11*t*-.

The SCDi-*t*11 (9*c*,11*t*-18:2/(9*c,*11*t*-18:2 + 11*t*-18:1)) decreased with the oil level and with C18 unsaturated FA intake (P = 0.013). The 9*c,*11*t*-18:2 in NL increased sharply and linearly with 11*t*-18:1 for the 4 treatments that presented a 10*t*/11*t* below 1 (i.e. 0% oil diets and the 4% oil/5% Cistus diet) (Fig. 3.1A, slope 0.472±0.0375, R^2 = 0.88, P < 0.0001). For the other treatments, a large variability was observed and the general pattern (Fig. 3.1B) suggests that as 11*t*-18:1 increase above about 20 mg/g of total FA in NL, the 9*c,*11*t*-18:2 fails to continue to increase. The SCDi-11*t* decreases as 10*t*-18:1 increase (slope, -0.08±0.027, R^2 = 0.36, P = 0.009).

Table 3.5 -Effects of *Cistus ladanifer* (CL) and vegetable oil (O) on biohydrogenation intermediates (mg/g of total FA) including CLA isomers (mg/100 g FA) present on neutral lipid fraction of longissimus muscle of lambs.

	0% Oil			4% Oil			8% Oil[1]			SEM	*P* values		
	5%CL	10%CL	20%CL	5%CL	10%CL	20%CL	5%CL	10%CL	20%CL		O	CL	O×CL
18:1 isomers													
6*t*-/7*t*-/8*t*-	1.8	1.8	2.0	2.5	2.9	4.2	3.7	3.9	4.6	0.37	<0.001	0.031	0.37
9*t*-	1.9	2.1	2.5	3.0	3.1	4.1	3.7	4.0	4.4	0.45	<0.001	0.008	0.60
10*t*-	5.7 ^{de} ±1.35	5.7 ^{e} ±1.35	6.3 ^{c} ±1.97	10.4 ^{cde} ±1.97	19.6 ^{bcd} ±1.97	37.7 ^{a} ±1.97	20.6 ^{bc} ±1.97	27.8 ^{ab} ±1.97	42.4 ^{abcde} ±12.4	-	0.29	0.74	0.035
11*t*-	14.5	13.4	16.5	20.2	18.1	22.5	22.7	23.3	22.2	2.67	0.015	0.64	0.88
12*t*-	3.0	2.8	3.7	4.7	4.7	5.8	5.5	6.8	5.6	0.58	0.001	0.46	0.35
15*t*-	5.8	3.6	5.7	6.0	5.1	5.6	7.3	7.5	6.3	0.64	0.010	0.26	0.23
12*c*-	2.4	2.6	3.5	4.0	4.4	4.0	4.2	5.7	4.0	0.55	0.010	0.35	0.33
13*c*-	1.6	1.7	1.8	2.8	1.9	1.7	2.2	2.4	1.9	0.43	0.34	0.49	0.55
16*t*-	2.1	1.9	2.2	3.4	2.8	3.1	3.5	4.0	2.9	0.52	0.002	0.52	0.43
16*c*-	0.6	0.6	0.5	1.1	0.9	1.3	1.3	1.4	1.4	0.15	<0.001	0.41	0.46
total	39.3	36.2	44.5	57.9	63.5	90.0	74.7	86.7	95.0	7.22	<0.001	0.025	0.40
18:2 isomers													
nonconjugated													
9*c*,13*t*-/8*t*,12*c*-	3.6	3.3	3.8	6.0	5.3	5.7	6.3	6.9	5.3	0.67	0.002	0.81	0.52
8*t*,13*c*-/ 9*c*,12*t*-	2.1	2.1	2.3	3.5	3.1	3.0	3.5	4.2	3.3	0.45	0.008	0.82	0.67
9*t*,12*c*-	0.4	0.5	0.6	1.0	1.0	1.2	1.4	1.7	1.9	0.20	<0.001	0.28	0.89
10*t*,15*c*/11*t*,15*c*-	3.0±1.1	3.2±1.0	3.5±1.0	8.1±1.0	10±1.0	16±1.0	16±1.7	20±1.0	25±2.2	-	0.001	0.038	0.21
*c*12,*c*15-	0.4	0.5	0.3	1.0	1.4	1.3	1.4	1.9	2.3	0.27	<0.001	0.26	0.53
total	10.4	10.6	12.2	23.1	23.2	30.7	32.4	38.1	41.8	2.54	<0.001	0.048	0.52
conjugated													
12*t*,14*t*-	12	10	8	16	18	26	23	32	29	3.5	<0.001	0.33	0.28
11*t*,13*t*-	14	12	14	21	21	39	29	32	28	4.0	0.001	0.22	0.10
10*t*,12*t*-	3^{c}±0.8	4^{c}±0.1	4^{c}±0.1	4^{c}±0.1	5^{c}±0.6	13^{a}±0.6	9^{b}±0.6	10^{b}±0.6	15^{a}±4.4	-	0.048	0.17	0.017
9*t*,11*t*-	36	23	38	46	27	35	44	42	47	11.0	0.44	0.44	0.92
8*t*,10*t*-	1±0.1	1±0.3	2±0.3	1±0.3	1±0.3	2±0.3	2±1.0	2±0.3	3±1.0	-	0.11	0.16	0.88
7*t*,9*t*-	5±1.1	4±1.0	7±1.0	7±1.0	5±0.1	6±1.0	8±3.6	8±1.0	9±3.7	-	0.47	0.57	0.68
12,14(*c*/*t*)-	7±2.6	9±2.4	8±2.4	20±2.4	30±8.4	33±8.4	36±8.4	62±8.4	58±9.2	-	<0.001	0.07	0.35
11*t*,13*c*-	23	21	26	34	34	45	50	49	39	6.0	0.003	0.94	0.37

11*c*,13*t*-	4±0.3	3±0.6	4±0.3	6±0.3	4±0.3	5±0.3	8±0.3	5±0.3	5±0.3	-	0.16	0.44	0.82
10*t*,12*c*-	5^{c}±1.0	5^{c}±1.0	6^{c}±1.0	8^{c}±3.1	15^{c}±3.1	43^{ab}±4.1	30^{b}±4.1	33^{ab}±4.1	48^{a}±4.8	-	<0.001	0.003	0.022
9*c*,11*t*-	667	603	704	881	677	744	733	846	778	117	0.39	0.86	0.71
8*t*,10*c*-	14	13	13	19	12	13	14	15	17	2.3	0.55	0.51	0.37
7*t*,9*c*-	36^{c}	40^{c}	43^{c}	50^{c}	87^{b}	111^{a}	84^{b}	107^{a}	112^{a}	5.5	<0.001	0.001	0.014
total	839	757	885	1124	945	1123	1078	1251	1194	130	0.029	0.74	0.73
18:3 isomers													
9*c*,11*t*,15*c*-	1.2	1.0	1.2	1.4	1.1	1.1	1.5	1.3	1.2	0.19	0.37	0.39	0.88
Total BI[2]	59	55	67	94	97	133	119	138	149	9.5	<0.001	0.040	0.48
10*t*/11*t* ratio[3]	0.39	0.51	0.37	0.58	1.21	1.71	0.89	1.25	1.83	0.19	0.001	0.008	0.11
SCDi-11*t*[4]	0.31	0.30	0.30	0.30	0.28	0.25	0.25	0.27	0.26	0.02	0.024	0.46	0.50

[1] Soybean and linseed oil blend (1:2 vol/vol); [2] Total Biohydrogenation Intermediates; [3] 10*t*-18:1/11*t*-18:1; [4] SCDi-11*t*= 9*c*,11*t*-18:2/(11*t*-18:1+9*c*,11*t*-18:2); [a,b,c,d,e] Values within a row with different superscripts differ significantly at $P < 0.05$

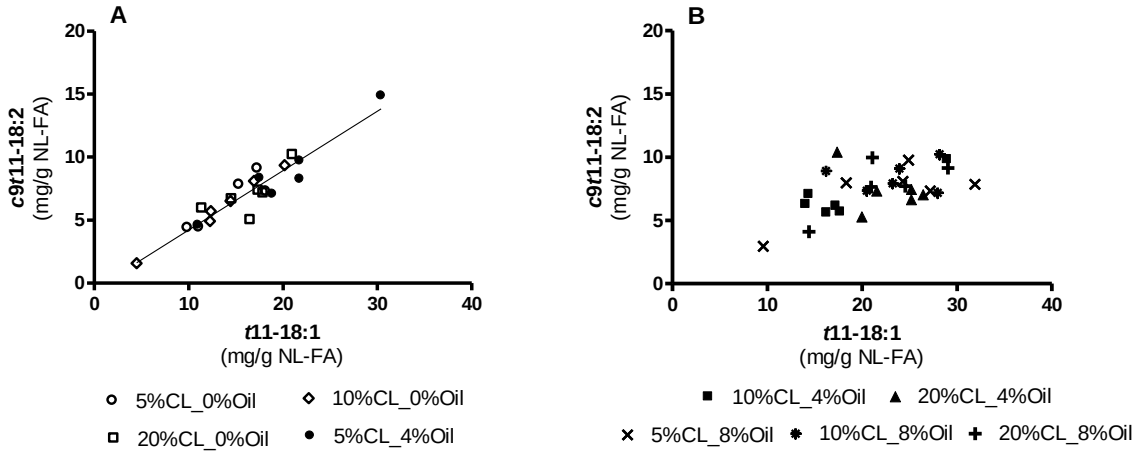

Figure 3.1- Relationship of 9*c*,11*t*-18:2 and 11*t*-18:1 concentration in muscle neutral lipids (NL). A – Data values from treatments that resulted in a 10*t*-/11*t*-18:1 ratio in NL below 1. B – Data values from treatments that resulted in a 10*t*-/11*t*-18:1 ratio in NL above 1.

3.3.2.2. Fatty acid composition of muscle polar lipids

The FA composition of PL is presented in Tables 3.6 and 3.7. The main FA in PL for lambs fed with diets without oil was 9*c*-18:1, while for lambs fed with diets with 4 and 8 % oil was the 18:2 n-6. Oil supplementation reduced the proportions of 9*c*-16:1, 17:0, 9*c*-17:1, 9*c*-18:1, 20:4 n-6, 22:4 n-6 and 20:3 n-9 and increased the proportions of 18:2 n-6, 18:3 n-3 and 20:3 n-3 (Table 3.6). For BI (Table 3.7), 6*t*/7*t*/8*t*-18:1, 9*t*-18:1, 11*t*-18:1, 12*t*-18:1, 12*c*-18:1, 16*c*-18:1, 12*c*,15*c*-18:2 and the conjugated dienes 10*t*,12*c*-, 7*t*,9*c*-18:2 increased with oil level in the diet. Thus, the sum of total 18:1 isomers and of non-conjugated 18:2 isomers increased in PL with oil supplementation. The inclusion of *C.ladanifer* had only minor effects of FA composition of PL, decreasing the 9*c*-16:1 and 22:6 n-3 and increasing the 6*t*/7*t*/8*t*-18:1, 11*t*,13*t*-18:2, 11*c*,13*t*-18:2, and 7*t*,9*c*-18:2.

Complementary regression analysis was applied to seleted FA, as described for NL. (Table 3.9). Independently of the C18 unsaturated FA intake, the CT intake increased the 7*t*,9*c*-18:2 and tended to decrease (P = 0.07) the 20:5 n-3, where for all the other FA that were significantly affected by the dietary Cistus (9*c*-16:1, 18:2 n-6, 22:6 n-3, 6*t*/7*t*/8*t*-18:1 and 11*t*,13*t*-18:2) were not significantly affected by CT intake, after adjustment for the C18 unsaturated FA intake.

Table 3.6 - Effects of *Cistus ladanifer* (CL) and vegetable oil (O) on polar lipid (PL) content and fatty acid composition of longissimus muscle of lambs

	0%Oil			4%Oil			8%Oil[1]			SEM	*P* values		
	5%CL	10%CL	20%CL	5%CL	10%CL	20%CL	5%CL	10%CL	20%CL		O	CL	O×CL
PL	12.4	12.1	12.2	13.8	13.8	12.7	12.9	13.3	14.3	0.89	0.22	0.99	0.69
Total FA in PL	2.92	2.82	2.69	3.48	3.08	2.87	3.11	3.03	3.38	0.34	0.39	0.73	0.78
14:0	5.0	6.8	4.8	5.6	6.0	5.3	4.4	6.6	7.4	1.23	0.82	0.40	0.62
a-15:0	1.3	1.3	0.8	1.1	1.4	1.3	1.1	1.1	1.3	0.28	0.83	0.88	0.67
15:0	2.8	2.5	2.4	2.2	2.5	2.2	2.2	2.5	2.7	0.29	0.53	0.94	0.54
16:0	173±24	156±7.9	150±7.9	139±7.9	141±1.8	137±7.9	132±8.6	145±7.9	141±8.6	-	0.29	0.75	0.86
i-17:0	3.4	3.0	3.9	2.8	4.0	4.2	3.9	3.5	3.3	0.34	0.68	0.34	0.10
7*c*-16:1	2.2	2.3	1.7	1.9	2.0	2.3	1.9	2.2	2.2	0.48	0.99	0.92	0.82
9*c*-16:1	9.5	9.1	6.7	6.0	5.7	4.5	4.6	5.2	4.7	0.51	<0.001	0.012	0.20
a-17:0	1.3±0.02	0.9±0.08	1.4±0.50	1.2±0.18	1.4±0.10	1.0±0.10	1.1±0.02	1.4±0.50	1.4±0.12	-	0.89	0.96	0.29
17:0	9.3	8.1	8.2	7.1	7.5	7.2	6.1	6.6	6.8	0.60	0.007	0.96	0.52
i-18:0	1.4	1.0	1.0	1.0	1.2	1.1	1.2	0.8	1.3	0.15	0.96	0.48	0.18
9*c*-17:1	6.2	5.8	6.4	5.4	5.4	5.0	4.1	4.1	4.4	0.70	0.030	0.94	0.96
18:0	107±13	101±2.6	93±2.6	92±4.9	96±2.6	96±4.9	97±2.9	99±4.9	96±5.5	-	0.61	0.64	0.76
9*c*-18:1	202	208	199	139	158	150	126	133	114	10.4	<0.001	0.33	0.86
11*c*-18:1	33	30	27	30	29	25	32	34	31	2.64	0.20	0.20	0.86
18:2 n-6	151	152	177	185	193	206	212	217	220	7.8	<0.001	0.06	0.69
18:3 n-3	17^{c}	18^{c}	19^{c}	47^{ab}	44^{b}	53^{a}	59^{a}	60^{a}	53^{a}	2.28	<0.001	0.87	0.07
20:0	1.3±0.06	1.4±0.12	1.6±0.12	1.4±0.25	1.3±0.12	1.7±0.12	1.3±0.13	1.2±0.25	1.7±0.27	-	0.87	0.10	0.81
11*c*-20:1	1.5	1.6	1.3	1.4	1.4	1.4	1.4	1.2	1.3	0.12	0.48	0.64	0.60
20:2 n-6	2.4	2.0	1.9	2.8	2.4	2.3	2.4	2.3	2.9	0.34	0.26	0.47	0.60
20:3 n-9	9.6	9.4	9.2	8.5	6.0	4.9	6.9	4.9	4.5	1.19	0.007	0.14	0.70
20:3 n-6/22:0	6.61^{ab}	6.36^{ab}	7.55^{a}	6.41^{ab}	5.68^{ab}	4.58^{b}	5.13^{ab}	4.32^{b}	4.60^{b}	0.443	<0.001	0.28	0.09
20:3 n-3	1.0	0.8	0.7	1.7	1.5	1.6	2.0	1.8	2.1	0.14	<0.001	0.34	0.61
20:4 n-6	68	66	62	62	51	44	47	40	43	5.8	0.006	0.20	0.70
20:5 n-3	20.5	21.6	18.4	28.1	21.3	19.3	25.3	18.8	17.7	2.68	0.44	0.054	0.57
22:4 n-6	4.4	4.5	4.2	3.8	3.2	3.2	2.1	2.1	2.3	0.392	0.001	0.76	0.76
22:5 n-3	21.4	23.5	21.9	27.8	23.4	20.0	21.2	18.9	17.6	2.11	0.08	0.14	0.40
22:6 n-3	7.9	6.9	5.7	8.1	7.3	5.2	6.0	4.4	4.2	0.97	0.054	0.045	0.89

[1] Soybean and linseed oil blend (1:2 vol/vol); [a,b,c] Values within a row with different superscripts differ significantly at $P < 0.05$

Table 3.7 - Effects of *Cistus ladanifer* (CL) and vegetable oil (O) on biohydrogenation intermediates (mg/g of total FA) including CLA isomers (mg/100 g FA) present on polar lipid (PL) fraction of longissimus muscle of lambs.

	0%Oil			4%Oil			8%Oil[1]			SEM	*P* values		
	5%CL	10%CL	20%CL	5%CL	10%CL	20%CL	5%CL	10%CL	20%CL		O	CL	O×CL
18:1 isomers													
6*t*-/7*t*-/8*t*-	1.0	1.2	1.1	1.0	1.3	1.6	1.4	1.7	2.1	0.18	0.011	0.031	0.53
9*t*-	1.4	1.6	1.4	1.4	1.9	2.3	2.1	2.3	2.9	0.27	0.008	0.09	0.39
10*t*-	4.0±1.42	4.7±1.36	3.7±1.36	5.3±1.36	8.1±2.95	17.6±2.9	9.8±3.03	13.8±2.9	21.8±8.4	-	0.06	0.25	0.25
11*t*-	4.3	6.1	5.7	5.9	9.0	11.3	10.2	10.6	8.6	1.30	0.006	0.23	0.20
12*t*-	2.4±0.08	2.8±0.06	2.7±0.06	2.9±0.38	3.3±0.38	3.0±0.63	3.6±0.42	4.6±0.63	4.3±0.68	-	0.015	0.23	0.92
12*c*-	5.0	6.3	7.9	7.8	8.5	7.4	10.0	11.6	8.7	1.06	0.008	0.36	0.30
13*c*-	1.2	1.3	1.0	1.4	1.4	1.8	1.6	1.8	1.3	0.18	0.046	0.62	0.26
16*t*-	1.2±0.22	1.1±0.19	0.8±0.02	1.0±0.19	1.4±0.19	2.2±0.11	1.8±0.22	1.4±0.19	1.4±0.22	-	0.27	0.88	0.45
16*c*-	0.9	1.0	0.9	1.1	1.2	1.6	1.3	1.4	1.5	0.12	0.003	0.09	0.31
total	20.7	26.1	25.2	27.7	36.2	48.8	41.8	48.9	51.2	4.03	<0.001	0.024	0.34
18:2 isomers													
- nonconjugated													
9*c*,13*t*-/8*t*,12*c*-	2.4	2.3	1.9	2.3	2.8	2.6	2.7	2.8	2.1	0.25	0.15	0.14	0.42
8*t*,13*c*-/9*c*,12*t*-	1.4	1.3	1.3	1.1	1.6	1.4	1.8	2.1	1.3	0.26	0.20	0.28	0.43
9*t*,12*c*-	1.3	1.1	1.1	1.0	1.0	0.9	1.1	1.1	1.1	1.03	0.08	0.21	0.76
10*t*,15*c*/11*t*,15*c*-	1.4±0.39	2.4±0.36	1.1±0.36	2.5±0.36	3.6±0.83	5.6±0.83	5.3±0.86	6.5±0.36	7.5±3.78	-	0.10	0.33	0.33
9*c*,15*c*-	3.1	2.1	1.6	2.0	1.8	2.1	3.2	3.9	3.2	1.35	0.41	0.91	0.96
12*c*,15*c*-	1.3±0.21	0.8±0.18	0.8±0.18	1.4±0.18	1.6±0.18	1.7±0.18	1.5±0.21	2.2±0.18	1.9±0.43	-	0.020	0.79	0.24
total	11.7	9.91	7.75	10.4	11.3	14.3	15.5	18.4	18.9	2.53	0.016	0.86	0.58
- conjugated													
12*t*,14*t*-	6±3.7	5±0.7	9±3.6	11±3.6	11±3.6	15±3.6	10±0.8	16±3.6	23±9.4	-	0.18	0.41	0.79
11*t*,13*t*-	4^{d}	5^{d}	7^{d}	12^{cd}	14^{bc}	21^{a}	21^{ab}	22^{a}	18^{abc}	1.1	<0.001	0.04	0.018
10*t*,12*t*-	2±0.9	2±0.1	4±0.8	4±0.8	3±0.8	5±0.8	4±0.9	5±0.8	16±2.9	-	0.18	0.22	0.41
9*t*,11*t*-	22±12.5	18±4.7	40±12.0	24±9.1	23±4.7	19±12.0	23±4.8	25±4.7	24±12.5	-	0.85	0.76	0.72
8*t*,10*t*-	8±3.2	5±1.7	14±10.0	10±3.2	4±1.7	4±1.7	5±3.2	8±1.7	15±11.0	-	0.63	0.45	0.61
7*t*,9*t*-	6±1.3	4±1.3	10±6.1	7±2.1	5±1.3	4±1.3	4±1.3	6±1.3	8±6.1		0.78	0.62	0.66
12,14(*c*/*t*)-	24±7.7	10±4.1	6±4.1	7±0.1	18±4.1	11±4.1	15±4.2	16±4.1	22±7.7	-	0.39	0.89	0.24
11*t*,13*c*-	8±1.6	8±3.1	10±3.1	13±1.4	15±3.1	22±3.1	28±12.7	22±3.1	20±3.4	-	0.15	0.77	0.66
11*c*,13*t*-	5^{c}	5^{c}	7^{bc}	7^{bc}	9^{bc}	12^{ab}	10^{abc}	15^{a}	11^{abc}	0.009	<0.001	0.04	0.06
10*t*,12*c*-	4±0.7	6±0.5	4.7±0.5	6±0.5	10±1.8	32±7.4	24±7.7	22±1.8	34±7.7	-	0.008	0.10	0.16

9*c*,11*t*-	204	224	259	233	268	245	228	266	206	24.1	0.58	0.34	0.41
8*t*,10*c*-	7±1.3	5±1.1	9±3.5	7±3.5	4±1.1	4±1.1	6±1.1	6±1.1	10±3.9	-	0.55	0.40	0.71
7*t*,9*c*-	10	14	14	14	21	23	17	25	25	2.0	0.001	0.006	0.65
total	315	318	397	358	413	425	396	462	428	42.8	0.11	0.28	0.76
Total BI[2]	78±2.9	81±2.6	73±2.6	81±5.7	88±5.7	98±5.7	101±6.0	112±2.6	110±18	-	0.12	0.47	0.44

[1] Soybean and linseed oil blend (1:2 vol/vol) ;[2] Total Biohydrogenation Intermediates; [a,b,c,d] Values within a row with different superscripts differ significantly at $P < 0.05$

3.3.3. Gene expression of *SCD*, *FADS1* and *FADS2* in muscle

The relative mRNA expression levels of *SCD*, *FADS1* and *FADS2* in the *longissimus* muscle of lambs are presented in Table 3.8. Dietary level of *C. ladanifer* increased SCD mRNA expression level, in contrast to dietary oil level, which had no effect. An interaction between the level of oil and *C. ladanifer* was observed for *FADS2* ($P = 0.018$) and the same trend was observed for *FADS1* ($P = 0.051$). In both cases, the relative mRNA expression level increased with *C. ladanifer* level except for the more extreme diet, with 8% of oil and 20% of Cistus, where it was depressed.

Results of regression analysis for *FADS1*, *FADS2* and *SCD* mRNA expression level with CT and FA daily intake indicated that the intake of CT increased ($P = 0.019$) *FADS1* and tended ($P = 0.11$) to increase *SCD* mRNA expression (Table 3. 9).

The *SCD* mRNA expression correlated with the amount of total meat lipids ($r = +0.36$, $P = 0.009$), NL and NL FA ($r = +0.36$, $P = 0.010$) but not with ∆-9 desaturase products (mg/g FA in NL) or product/substrate ratios, except for 7*t*,9*c*-18:2 ($r = +0.34$, $P = 0.014$). The *SCD* expression was also positively correlated with *t*10 BI and 10*t*/11*t* ratio in NL ($r = +0.36$ $P = 0.009$). There was a positive correlation ($r = +0.79$, $P < 0.001$) between mRNA expression levels of *FADS1* and *FADS2*, suggesting that both genes are co-expressed. *FADS1* was also correlated with *SCD* ($r = +0.53$, $P < 0.001$). The *FADS2* mRNA expression was positively correlated with the percentage of 18:2 n-6 ($r= 0.34$, $P = 0.017$) and 18:3 n-3 ($r = 0.38$, $P = 0.007$) and negatively correlated with 20:4 n-6 ($r = -0.31$, $P = 0.037$) and with the sum of n-6 LC PUFA ($r = -0.30$, $P = 0.042$) in PL. The correlations for *FADS1* mRNA expression with PUFA followed a general pattern similar to the *FADS2*, but did not reach significance ($P > 0.05$).

Table 3.8 - Effects of *Cistus ladanifer* (CL) and vegetable oil blend (O) on relative mRNA expression level (arbitrary units x 10) of the codifying genes for Δ9 (*SCD*), Δ6 (*FADS2*) and Δ5 (*FADS1*) desaturases

	0%Oil			4%Oil			8%Oil[1]			SEM	*P* values		
	5%CL	10%CL	20%CL	5%CL	10%CL	20%CL	5%CL	10%CL	20%CL		O	CL	O×CL
SCD	25.8	27.0	48.1	21.9	63,6	73.3	30.6	34.2	52.2	9.53	0.101	0.015	0.292
FADS2	5.8^{c}	6.4^{bc}	9.9^{ab}	7.1^{bc}	7.7^{c}	11.5^{a}	10.6^{ab}	10.4^{ab}	6.2^{c}	1.10	0.229	0.356	0.018
FADS1	19.4	18.6	27.3	18.9	24.1	33.3	24.1	26.8	18.3	2.39	0.294	0.128	0.051

[1] Soybean and linseed oil blend (1:2 vol/vol)

[a,b,c,d] Values within a row with different superscripts differ significantly at $P < 0.05$

Table 3.9 - Regression models of selected fatty acids (mg/g FA) and mRNA expression levels of desaturase genes with condensed tannins intake (CTi, g/d) and C18 unsaturated FA intake (C18UFAi, g/d)

	Intercept		CTi		C18UFAi		CTi × C18UFAi		R^2	RMSE
	estimate±SE	*P* =	estimate±SE	*P* =	estimate±SE	*P* =	estimate±SE	*P* =		
Neutral Lipids										
17:0	14.9±0.60	<0.001	-0.08±0.033	0.036	-0.045±0.01	0.001	-	-	0.634	1.06
18:0	165.3±4.19	<0.001	-0.70±0.231	0.009	-0.26±0.075	0.003	-	-	0.631	7.39
6*t*/7*t*/8*t*-18:1	1.28±0.426	0.009	0.02±0.024	0.343	0.05±0.011	<0.001	-	-	0.615	0.75
9*t*-18:1	1.65±0.300	<0.001	0.02±0.017	0.220	0.04±0.007	<0.001	-	-	0.710	0.53
10*t*-18:1	9.6±6.46	0.16	-0.72±0.489	0.163	0.018±0.230	0.938	0.048±0.0169	0.013	0.785	7.35
11*t*-18:1	13.7±2.09	<0.001	0.015±0.116	0.893	0.18±0.053	0.042	-	-	0.463	3.68
9*c*-18:1	386±13.8	<0.001	-1.39±0.763	0.087	-0.91±0.250	0.002	-	-	0.633	1.06
11*c*-18:1	12.1±0.48	<0.001	-0.05±0.026	0.055	-0.02±0.009	0.041	-	-	0.427	0.84
18:2 n-6	22.4±1.92	<0.001	0.12±0.106	0272	0.18+0.035	<0.001	-		0.666	3.39
18:3 n-3	5.0±2.20	0.038	-0.02±0.122	0.851	0.30+0.039	<0.001	-	-	0.798	3.88
20:0	1.29±0.153	<0.001	0.04±0.008	<0.001	-0.003±0.003	0.239	-		0.566	24.4
10*t*,12*t*-18:2[1]	4.4±2.62	0.113	-0.21±0.263	0.304	-0.003±0.067	0.961	0.011±0.0049	0.048	0.662	0.03
NL FA[2]	9.6±1.72	<0.001	0.21±0.094	0.043	0.003±0.031	0.928	-	-	0.255	3.03

Polar Lipids										
18:2 n-6	150±8.60	<0.001	0.28±0.475	0.562	0.88±0.155	<0.001	-	-	0.696	15.2
18:3 n-3	17.7±4.92	0.003	-0.42±0.271	0.138	0.67±0.088	<0.001	-	-	0.789	8.68
6*t*/7*t*/8*t*-18:1	0.76±0.188	0.001	0.016±0.010	0.147	0.01±0.003	0.011	-	-	0.458	0.33
7*t*,9*c*-18:2[1]	7.7±0.177	<0.001	0.21±0.098	0.049	0.19±0.032	<0.001	-	-	0.759	3.13
20:5 n-3	22.9±2.60	<0.001	-0.27±0.144	0.070	0.024±0.047	0.621	-	-	0.195	4.59
20:6 n-3	7.4±1.01	<0.001	-0.06±0.055	0.278	-0.02±0.018	0.303	-	-	0.161	1.78
Gene expression										
SCD	16.1±3.05	<0.001	0.28±0.17	0.111	0.08±0.055	0.158	-	-	0.288	5.39
FADS2	5.6±1.28	<0.001	0.09±0.070	0.223	0.038±0.002	0.111	-	-	0.262	2.25
FADS1	17.8±9.93	0.093	1.43±0.548	0.019	0.16±0.179	0.399	-	-	0.365	17.5

[1] expressed in mg/100g of FA

[2] total FA in NL

3.4. Discussion

Our team reported previously that the inclusion of 25% of *C. ladanifer* to an all-forage basal diet supplemented with 6% of a vegetable oil blend resulted in a large increase of *t*11-18:1 in abomasal digesta (+100%) and muscle NL (+76%), and in a milder increase (+37%) of 9*c,*11*t*-18:2 in muscle NL (Jerónimo *et al.*, 2010b). Thus, in spite of large availability of substrate (11*t*-18:1) obtained previously with dietary inclusion of Cistus, we did not observed an equivalent increase of 9*c,*11*t*-18:2 in muscle, and this could be due to a down-regulation of SCD. The large majority of 9*c,*11*t*-18:2 deposited in muscle is expected to be derived from endogenous synthesis catalyzed by ∆-9 desaturase (Palmquist *et al.*, 2004, Gruffat *et al.*, 2008). The ∆-9 desaturase inhibition could be somehow due to Cistus secondary compounds. *Cistus ladanifer* is rich in CT, which might up regulate ∆-9 desaturase protein expression (Vasta *et al.*, 2009c) or activity (Rana *et al.*, 2012). Conversely, all-forage diets are known to down-regulate *SCD* expression (Daniel *et al.*, 2004) and activity (Smith *et al.*, 2009). Moreover, increased metabolic PUFA availability can also induce an inhibitory effect on ∆-9 desaturase activity (Daniel *et al.*, 2004). Thus, we hypothesized that using a basal diet containing a forage-to-concentrate ratio of 1:1, supplemented with vegetable oil and *C. ladanifer*, would allow a high 11*t*-18:1 metabolic availability and mitigate the forage ∆-9 desaturase down-regulation, resulting in higher 9*c,*11*t*-18:2 deposition in muscle. The present results indicated that a 1:1 forage-to-concentrate ratio basal diet does not increase 9*c,*11*t*-18:2 with any combination of oil and *C. ladanifer* levels. The reason for that were the changes in biohydrogenation pathways, resulting in the replacement of 11*t*-18:1 by 10*t*-18:1 as the major biohydrogenation intermediate (hereafter *trans*-10shift), observed when the *C. ladanifer* and oil increased in the diet. The occurrence of the *trans*-10 shift has been frequently reported in ruminants fed low-forage, high-oil diets (Aldai *et al.*, 2013) and in growing ruminants fed low-forage diets even without oil supplementation (Rosa *et al.*, 2014).
Polyunsaturated FA intake has a profound and well established effect on the accumulation of BI in rumen and tissues (Shingfield *et al.*, 2013; Shingfield & Wallace, 2014). Therefore, one note of caution must be made on the interpretation of the effects of dietary *C. ladanifer* on deposition of BI in meat, as the effects of dietary *C. ladanifer* and C18 unsaturated FA intake might be confounded due to changes in diet ingredients and to differences of voluntary feed intake. This is clearly perceived from the interaction between both main factors (*C. ladanifer* and Oil) on FA intake data, particularly if we take in consideration that *C. ladanifer* has a low content in PUFA (Guerreiro et al., 2015). For this reason, whenever a significant effect of *C. ladanifer* inclusion level was observed, it was confirmed by complementary regression analysis using the intake of CT and C18 unsaturated FA as independent variables.

The impact of biohydrogenation pathways on meat FA profile are better perceived in muscle NL lipids than in PL or whole muscle FA, as most of BI are preferentially deposited in TAG and not in membrane phospholipids (Jerónimo *et al.*, 2011). In the present experiment, although 11*t*-18:1 in NL increased linearly with unsaturated C18 FA intake, it was not affected by either CT intake or dietary *C. ladanifer* level. Conversely, 10*t*-18:1 and other *trans*-10 shift related BI like 10*t*,15*c*-18:2, 10*t*,12*t*-18:2 and 10*t*,12*c*-18:2 displayed a large increase in NL when oil and *C. ladanifer* levels increased in the diets. The response of 10*t*,15*c*-18:2 was confounded by the co-elution with 11*t*,15*c*-18:2 isomer (Alves & Bessa, 2014). The effect of the oil level on 10*t*-18:1 may be explained by the fairly high amount of cereals in the basal diet (Bessa *et al.*, 2005), but the clear positive interaction between CT and C18 PUFA intake was surprising. Condensed tannins have been frequently reported to increase the *trans*-18:1 isomers in the rumen and tissues, as reviewed by Vasta and Bessa (2012). The reason why increased rumen concentration of CT results in induced *trans*-18:1 accumulation is not clear. It can be either due to the suppression of microbial community able to conduct the last reductive step of biohydrogenation (Khiaosa-Ard *et al.*, 2009) or as rumen microbiota stress response (Bessa *et al.*, 2000). The increase of *trans*-18:1 isomers in ovine digesta, tissues, or milk induced by dietary tannins is mostly due to the 11*t*-18:1 increase when basal diet allows for the predominance of the usual 11*t* biohydrogenation pathways (Vasta *et al.*, 2009b; Jerónimo *et al.*, 2010b; Buccioni *et al.*, 2015). However, when dietary forage percentage decreases to about 40% of the diet, the increase of *trans*-18:1 induced by tannins was equally explained by both 10*t*-18:1 and 11*t*-18:1 (Toral, Hervas, Belenguer, Bichi & Frutos, 2013). When high-concentrate diets, even if not lipid supplemented, were fed to lambs, the increase of *trans*-18:1 induced by tannins was due mostly to the 10*t*-18:1 increase (Vasta *et al.*, 2009b). In the present experiment, despite a middle dietary forage inclusion (50%), the *C. ladanifer* tannins increased mostly the 10*t*-18:1 in NL, with no effect on 11*t*-18:1. The synergy between PUFA and tannins intake promoting the *trans*-10 shift is probably undesirable, assuming that 10*t*-18:1 has deleterious health effects to consumers, as reviewed by Aldai *et al.* (2013) and is not a substrate for endogenous CLA synthesis.

Despite the exuberant 10*t*-18:1 increase in NL, oil supplementation also increased 11*t*-18:1, but not 9*c,*11*t*-18:2. The 9*c,*11*t*-18:2 is expected to increase linearly with *t*11-18:1 in the tissues reflecting the Δ-9 desaturase activity (Daniel *et al.*, 2004; Palmquist *et al.*, 2004). The low amount of 9*c,*11*t*-18:2 found in abomasal digesta (data not shown) suggests that the rumen derived 9*c*,11*t*-18:2 might not have a relevant contribution to the amount of 9*c,*11*t*-18:2 found in NL. The failure of 9*c,*11*t*-18:2 to increase linearly with the 11*t*-18:1 concentration when the *trans*-10 shift is clearly established (Fig. 3.1) suggests that the Δ-9 desaturase activity is somehow blocked. It is not clear why this happened, but suggest that it

could be due to the accumulation of BI containing the 10*t* double bond, as it has been demonstrated that at least the 10*t*,12*c*-18:2 inhibits the Δ-9 desaturase activity in lamb tissues (Wynn *et al.*, 2006). We could also speculate that in the presence of high availability of 10*t*-18:1, the substrate binding sites of Δ-9 desaturase might be transiently occupied by the 10*t*-18:1, hampering the binding of Δ-9 desaturase substrates.

The *SCD* mRNA level in muscle was not related with any Δ-9 desaturase products or Δ-9 desaturase activity indices computed by product to substrate ratios. In fact, post-mortem *SCD* mRNA expression levels in muscle might not be the best predictor of the Δ-9 desaturase products content in meat and of the sustained Δ-9 desaturase activity during the duration of the finishing period, as discussed by Bessa *et al.* (2015). In the present experiment *SCD* mRNA expression levels increased with *C. ladanifer* level, although this was not clearly confirmed by regression analysis with CT intake (P = 0.11), suggesting that it might not be a direct effect of *C. ladanifer* tannins. However, a more clear effect of CT intake on *FASD1* mRNA expression levels was observed. Others, have reported an increase in Δ-9 desaturase protein expression (Vasta *et al.*, 2009c), or in Δ-9 desaturase activity (Rana *et al.*, 2012) associated with dietary tannins intake. The mode how CT could modulate gene expression is not clear at present. In Jerónimo *et al.* (2010b) our team reported that joint supplementation of *C. ladanifer* and vegetable oil increased the IMF of lambs. In the present experiment, the effect of dietary *C. ladanifer* inclusion on IMF was not evident, although NL and NL FA increase linearly with CT intake. The potential of dietary *C. ladanifer* to increase IMF should be better studied but could be linked to its putative effects on adipogenesis gene expression.

In ruminants, the dietary PUFA escaping the biohydrogenation and absorbed are preferentially incorporated in PL and cholesterol esters of intestinal lipoproteins in order to more easily supply the plastic PUFA requirements of the body membrane (Moore & Christie, 1984). Thus, the accumulation of C18-PUFA in meat NL (mostly triacylglycerols) should reflect their availability beyond the mandatory requirements for incorporation in membrane PL and thus can provide an indirect indication of the extent of PUFA rumen biohydrogenation. Our data suggests that the inclusion of *C. ladanifer* increases the C18 PUFA availability, as its concentrations increased in NL. This could be explained by an inhibition of C18-PUFA rumen biohydrogenation mediated by CT, similarly to what was described by others (Kronberg, Scholljegerdes, Barcelo-Coblijn & Murphy, 2007; Buccioni *et al.*, 2015). However, the linear increase of C18-PUFA in NL with CT intake was not confirmed by the regression analysis adjusted also for C18 unsaturated FA intake, indicating that *C. ladanifer* tannins probably did not reduce the extent of C18 PUFA biohydrogenation in the rumen.

The FA composition of PL is under strong regulatory control in order to maintain the functionality of cellular membranes (Bessa *et al.*, 2015) but even so changing dietary PUFA supply induce an extensive remodeling of PUFA in PL (Jerónimo *et al.*, 2011; Rosa *et al.*, 2014). Consistently to this, in the present experiment increasing oil in the diet resulted in an extensive replacement of 9*c*-18:1 by both 18:2 n-6 and 18:3 n-3. The BI were incorporated in much less extent in PL than in NL although the same general pattern found in NL can also be recognized in PL, which confirms previous observations (Jerónimo *et al.*, 2011; Rosa *et al.*, 2014).

In ruminant muscle, the very long chain PUFA are located almost exclusively in membrane phospholipids (Bessa *et al.*, 2015). In mammals the LC-PUFA are formed by elongation and desaturation (via Δ6 and Δ5-desaturases) of C18 PUFA (Alvarenga *et al.*, 2015). Increasing the n-3 LC-PUFA in meat is highly desirable, considering its beneficial health effects and the large dependence of marine foods sources to met the nutritional requirement of humans (Alvarenga *et al.*, 2015). Thus, the decrease of 20:5 n-3 and 22:6 n-3 in muscle PL associated with dietary *C. ladanifer* incorporation is negative. Nevertheless, the complementary regression analysis with CT and C18 UFA intakes, only weakly (P = 0.07) confirmed the decrease of the 20:5 n-3 with CT intake. The expression of *FADS1* (Δ5-desaturase gene) in muscle was positively associated with CT intake, which could be interpreted as a compensatory overexpression to couple with diminished n-3 LC-PUFA. Nevertheless, the depression of n-3 LC PUFA with *C. ladanifer* is small and was not present in the other experiment where *C. ladanifer* were fed to lambs (Jerónimo *et al.*, 2010b), thus it might not be a consistent and a biologically relevant effect.

Concluding, the inclusion of *C. ladanifer* in oil supplemented lamb diets containing 50% of concentrate has a negative effect on lamb meat FA profile, mainly due to the exacerbation of ruminal *t*10-shift, with a strong accumulation of *t*10-18:1 and no increase on 9*c*,11*t*-18:2. Moreover, the inclusion of *C. ladanifer* in these diets results in a slight reduction on the n-3 LC PUFA in lamb meat, which is an undesirable nutritional effect, due to the importance of these FA for human health.

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CHAPTER 4

Alternative feeds for lamb diets: the effects of inclusion of *Cistus ladanifer*, L. and the replacement of cereals by dehydrated citrus pulp on Merino Branco growth performance, carcass composition and meat quality

Alternative feeds for lamb diets: the effects of inclusion of *Cistus ladanifer*, L. and the replacement of cereals by dehydrated citrus pulp on Merino Branco growth performance, carcass composition and meat quality

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Contribution of Alexandra E. Francisco to this article:

Alexandra E. Francisco participated in the *C. ladanifer* harvesting, in the animal experiment and in the sample collection. Collaborated on the evaluation of meat quality parameters and performed the data processing and statistical analysis. Furthermore, Alexandra E. Francisco integrated the trained sensory analysis panel and participated in the interpretation and discussion of the results, as well as in the writing of the manuscript.

ABSTRACT

The effects of the inclusion of *Cistus ladanifer*, L. (*C. ladanifer*) and dehydrated citrus pulp (DCP) in diets with 50% forage for growing lambs were evaluated on growth performance, carcass and meat quality. Thirty two ram lambs were randomly assigned to 16 groups of 2 lambs each and to 4 diets, resulting from the combination of the two factors (Cistus 0 *vs* 150 g/kg DM, and Cereals *vs* DCP). Intake increased (P = 0.008) with *C. ladanifer* but growth was not affected by treatments. A significant interaction between the two factors was found for dressing percentage (P = 0.041). Diets did not influence the risk of rumen parakeratosis, carcass composition, muscle pH, cooking losses, meat colour and stability after 7 days of storage. Both Cistus and DCP affected tenderness and juiciness, and *C. ladanifer* reduced (P < 0.001) overall acceptability. *Cistus ladanifer* reduced the content of meat in *trans* fatty acids (TFA). Dehydrated citrus pulp slightly improved meat nutritional value, increasing the content on 18:3 n-3.

Key words: lamb, *Cistus ladanifer,* dehydrated citrus pulp*,* carcass quality, meat quality

4.1. Introduction

Cistus ladanifer (rockrose) is an aromatic shrub frequent in the Mediterranean ecosystems, with high levels of plant secondary metabolites, such as phenolic compounds and condensed tannins (CT) (Guerreiro *et al.*, 2016). Although *C. ladanifer* presents low nutritive value and low acceptability by grazing ruminants, it can be included up to 10% in lamb diets where forage represented 50 % DM (Francisco *et al.*, 2015) or even more (25% DM) in all forage diets (Jerónimo *et al.*, 2010b, 2012), without negative effects in growth performances, carcass and meat quality traits. In a previous experiment it was observed that *C. ladanifer* exerted a synergic effect with oil on the pattern of rumen biohydrogenation derived isomers present in meat, enhancing the deposition of beneficial health FA such as 11*t*-18:1 and 9*c*,11*t*-18:2 (Jerónimo *et al*., 2010b). Moreover, dietary *C. ladanifer* is able to decrease the meat lipid oxidation (Jerónimo *et al.*, 2012; Francisco *et al.*, 2015) and improve the colour stability during storage (Francisco *et al*., 2015). Antimicrobial activity is also associated with *C. ladanifer* essential oil and extracts (Greche *et al.*, 2009; Barrajón-Catalán *et al.*, 2010).

Citrus pulp is an agro-industrial by-product of the juice industry that is widely available for the industry of compound feeds for ruminants as dehydrated pellets (DCP). The main characteristics of DCP are a low starch and protein content and a high level of pectins and sugars that are readily and highly fermentable in the rumen. It presents a NDF level intermediate between most concentrates and forages (Bampidis & Robinson, 2006). Citrus pulp can replace grain or forage in small ruminant diets without compromising animal

performance (Bhattacharya & Harb, 1973; Rodrigues *et al*., 2008a; Bueno *et al.*, 2004) or meat quality (Lanza *et al*., 2001; Scerra *et al.*, 2001; Caparra *et al.*, 2007; Rodrigues *et al*., 2008b). However, some authors have referred that high levels of DCP can increase the probability of ruminants to develop rumen parakeratosis, with negative impact in animal performance (Pascual & Carmona, 1980; Bampidis & Robinson, 2006).

Citrus pulp also present high levels of bioactive compounds with antioxidant activity, such as polyphenols, terpenes, carotenoids and vitamin C (Abeysinghe *et al*., 2007; Tripoli *et al*., 2007) and polyunsaturated fatty acids (PUFA) (Rodrigues *et al*., 2010). The beneficial effect of dietary citrus pulp on meat oxidative stability (Inserra *et al*., 2014) and on meat FA composition (Lanza *et al*., 2015) have been recently reported.

Meat from ruminants fed with high-concentrate diets is more susceptible to oxidation, , due to their low content on antioxidant substances and high on pro-oxidant components, especially if diets are lipid supplemented (Luciano *et al.*, 2012). Therefore, the inclusion of feed sources with, high levels of bioactive components with antioxidant activity, on concentrates for ruminants may be an important nutritional strategy to improve meat oxidative stability.

In the present trial was evaluated the combined effects of *C. ladanifer* and DCP inclusion in the diet of Merino Branco lambs,on productivity, carcass composition, meat quality. In a companion paper the results obtained for intramuscular fat composition will be reported.

4.2. Materials and methods

4.2.1. Animals and management

The animal handling followed EU Directive 2010/63/UE concerning animal care. Thirty-two MB ram lambs born in autumn 2013 were reared with dams on extensive grazing until weaning at approximately 60 days of age. At weaning, lambs were transported to the Polo de Investigação da Fonte Boa, Instituto Nacional de Investigação Agrária e Veterinária (Fonte Boa-INIAV), located at Vale de Santarém, Portugal, where the trial was conducted. Lambs were housed and randomly assigned to 16 pens; 2 lambs per pen and 4 pens per treatment, according to a completely randomized experimental design with a 2×2 factorial arrangement of treatments. The first factor was the dietary inclusion of *C. ladanifer* (0 *vs*. 150 g/kg DM) replacing partially the dehydrated lucerne, and the second factor was the dietary inclusion of DCP replacing totally the cereals (Cereals *vs.* DCP). According to this, the experimental diets were: 1) C0 – cereals and 0% *C. ladanifer*; 2) C15 - cereals with 15% of *C. ladanifer*; 3) DCP0 - dehydrated citrus pulp and 0% *C. ladanifer*; 4) DCP15 - dehydrated citrus pulp with 15% of *C. ladanifer*. All diets included 60 g/kg of soybean oil. All the diets presented 1:1 for

forage to concentrate ratio. Leaves and soft stems of *C. ladanifer* shrubs were harvested in Portugal (39°30'36"N/8°19'00''W) in April 2013, dried at room temperature, cut in small pieces, and milled to a final particle size of 3 mm. Diets were prepared and milled in the Fonte Boa-INIAV. The ingredient composition of the four diets and the chemical composition, obtained as the average of the results of three pooled samples of each diet, are presented in Table 4.1.

Table 4.1 – Proximal (%) and chemical composition (g/kg DM) of the experimental diets.

	0% *Cistus ladanifer*		**15% *Cistus ladanifer***	
	Cereals	**Citrus Pulp**	**Cereals**	**Citrus Pulp**
Ingredients				
Maize	5.00	0.00	5.00	0.00
Wheat	21.5	0.00	20.00	0.00
Soybean meal 42 %	15.0	17.0	17.5	19.5
Dehydrated citrus pulp	0.00	24.5	0.00	23.0
Dehydrated lucerne	50.0	50.0	35.0	35.0
Cistus ladanifer	0.00	0.00	15.0	15.0
Soybean oil	6.00	6.00	5.00	5.00
Sodium bicarbonate	0.50	0.50	0.50	0.50
Calcium carbonate	1.30	1.30	1.30	1.30
Minerals and vitamins	0.30	0.30	0.30	0.30
Salt	0.40	0.40	0.40	0.40
Chemical composition				
Dry matter (%)	89.8	89.8	89.6	89.5
Crude protein	171	178	168	170
NDF[1]	331	337	303	334
Starch	225	37	209	34
Ether extract	81	78	80	80
Ash	95	110	83	109
Calcium	14	17	15	18
Phosphorus	3.1	2.9	3.2	2.8
Total phenols[2]	6.46	10.2	18.1	27.5
Condensed tannins	0.40	0.76	3.53	5.58
Gross energy (Mj/kg)	18.37	18.32	18.75	18.52
Fatty acid (% of total fatty acids)				
16:0	14.7	14.8	15.1	14.6
18:0	4.41	4.73	4.64	4.63
9*c*-18:1	23.6	23.0	22.7	22.8
18:2 n-6	50.3	49.5	50.5	50.1
18:3 n-3	7.00	7.98	7.00	7.87

[1] – neutral detergent fiber; [2]– mg acid tannic equivalents/kg DM

During 7 days of adaptation to the experimental conditions, the lambs were dewormed by dosing with Ivomec® (Merial Labs.,Spain) and vaccinated against enterotoxaemia (Miloxan,

Merial Labs.,Spain). At the beginning of trial, the lambs were weighed and the average live weight (LW) obtained was 18.8 ± 3.44 kg (mean ± S.D). The trial lasted for 6 weeks, being the feed offered daily at 9:00 am at a rate of 110% of *ad libitum* intake calculated by weighing-back refusals daily which were registered and discarded. The animals were weighed weekly just before feeding.

4.2.2. Slaughter, carcasses evaluation and sample collection

At the end of the trial, lambs were weighed and transported to the experimental abattoir of the Fonte Boa-INIAV, where they were stunned and slaughtered by sectioning jugular veins and the carotid arteries. The evaluation of ruminal parakeratosis was conducted immediately after evisceration, by a visual grading of 4 scores of parakeratosis intensity (0 - normal rumen papillae, 1 - weak, 2 - moderate and 3 - strong) according to Tamate, Nagatani, Yoneya, Sakata & Miura (1973). After preparation, the carcasses were immediately weighed to obtain hot carcass weight (HCW), and were kept in a refrigerated room at 10 °C for 24 h, to prevent cold shortening. After that period, carcasses were re-weighed, to obtain the cold carcass weight (CCW) and graded for conformation and fat cover, using the EUROP classification systems, for lamb carcasses (EC, 2011a and EC, 2011b). Then, carcasses were chilled at 2 °C until the third day after slaughter, when the kidney knob channel fat (KKCF) and kidneys were removed and weighed, the carcasses were split along the spine and the left sides were separated into eight joints, as described in Santos-Silva et al. (2002b). Each joint was weighed to estimate the proportion of the higher-priced joints (leg+chump+loin+ribs). Chump and shoulders were vacuum-packed and frozen at -20 °C, until dissection for the evaluation of the carcass tissue composition.

In the left halves of the carcasses the loin joints containing the *Longissimus lumborum* muscle, were vacuum-packed and frozen at -20 °C until shear force determination. In the rib joints, the *Longissimus thoracis* muscle was isolated and two samples with about 1 cm thickness were collected for the evaluation of the lipid and colour stability after 7 days of storage. One sample was used at day 0 for the evaluation of colour parameters that were determined after 1 h of blooming. The other sample was individually placed on a polystyrene tray, over-wrapped with oxygen permeable polyvinyl chloride film and stored during 7 days in a refrigerator at 2 °C temperature and under permanent light. After the end of storage period, meat colour parameters were determined and the samples were vacuum packed and stored at -80 °C until lipid oxidation analysis. The remaining portions of the *Longissimus thoracis* muscle, were minced with a food processor (3×5 s) after the removal of the *epimysium*, and then were vacuum-packed and stored at -20 °C until further analysis for chemical composition.

In the right halves of the carcasses, the loin joints, containing *Longissimus lumborum* were vacuum packed and frozen at -20 °C, until being used for sensory analysis.

4.2.3. Analytical procedures

4.2.3.1. Feed

Diets were analysed for dry matter (DM) (ISO 6496, 1999), ash (ISO 5984, 2002), crude protein (ISO 5983, 1997), ether extract (ISO 6492, 1999), calcium (ISO, 6869/2007) and phosphorus (NP874/2000). Neutral detergent fibre was assayed according to Van Soest *et al.* (1991) with sodium sulphite, without alpha amylase and expressed with residual ash method. Starch was determined according to Clegg (1956). Fatty acid methyl esters of feed were prepared according to the method of Sukhija and Palmquist (1988), with toluene and nonadecanoic acid (19:0) as internal standard. Diet gross energy content was measured using an adiabatic bomb calorimeter (Parr 1261, Parr Instrument Company, USA).

The extraction and analysis of phenolic compounds were carried out as described by Khazaal *et al*. (1993). Total phenols content was determined by Folin–Ciocalteu's assay according to Julkunen-Tiito (1985), using tannic acid as standard and the concentration expressed as g tannic acid equivalent/kg DM. Total extractable CT were measured using butanol-HCl method (Porter *et al.*, 1986). The concentration of CT in the diets was quantified using *Cistus ladanifer* purified CT as standard.

4.2.3.2. Meat

4.2.3.2.1. Colour, pH, shear force and cooking loss

Meat colour was measured using a Minolta CR-300 Chromometer (Konica Minolta, Portugal) employing the CIE L^*, a^*, b^* system, where L^* is lightness, a^* redness and b^* yellowness. Measurements were made using the C illuminant and 2º standard observers. It was also calculated the Hue angle (H*) as $\tan^{-1}(a^*/b^*) \times (180/\pi)$ and colour saturation (Chroma, C*) as $(a^{*2} + b^{*2})^{½}$. For the evaluation of the colour changes during storage were determined the following indexes: $\Delta E_{(7-0)}=((\Delta L^*_{(7-0)})^2+(\Delta a^*_{(7-0)})^2+(\Delta b^*_{(7-0)})^2)^{0.5}$, $\Delta H_{(7-0)}$=(Hue value day 7) – (Hue value day 0) and $\Delta C_{(7-0)}$= (Chroma value day 7) – (Chroma value day 0).
Muscle pH was determined according to ISO2917/1999 where 5 g of meat from *Longissimus thoracis* was suspended and homogenized in 50 ml of a solution of potassium chloride 0.1 M. Then, pH was measured using a pH meter 744 Metro equipped with a combined glass electrode. To determinate meat shear force, the frozen left loins were thawed for 24 h at 2

°C, and after that, *Longissimus lumborum* muscle was isolated and cooked in an electric oven at 170 °C until reaching the internal temperature of 70 °C, which was monitored by an internal thermocouple (Thermometer, Eomega RDXL4SD, Manchester, USA). Cooking loss was determined as the difference in meat sample weights before and after cooking, expressed as a percentage of initial weight. After cooking, muscle samples were kept at 4 °C for 24 h, and then, each sample was cut in the direction of the fibres, in subsamples with a section of about 1 cm^2 and 2 cm long. Shear force (kgF) was determined using a Warner-Bratzler shear device, in a Texture Analyser (TA-XT2i) (TA-tx2i Texture Analyser, Stable Micro Systems, Surrey, UK), equipped with a load cell of 25 kg. A crosshead speed of 2 mm/s, along 25 mm was used. Data were collected using specific software (Texture Expert Exceed, Stable Micro Systems, Surrey, UK). For each loin, was recorded the maximum force needed to shear the samples perpendicularly to the axis of the fibre direction, expressed as the average of a minimum of 12 and a maximum of 27 determinations.

4.2.3.2.2. Nutritional composition of meat intramuscular fat

Meat was analysed for DM (ISO 1442, 1997) and lipid composition. Intramuscular lipids were extracted according to the procedure Folch, Lees, and Stanley (1957), but using dichloromethane and methanol (2:1 v/v) instead of chloroform. Fatty acids were transesterified according to Raes, De Smet, and Demeyer (2001), using sodium methoxide in methanol, followed by hydrochloric acid in methanol (1:1 v/v). For the analysis of methyl esters was used a chromatograph HP6890A (Agilent, Avondale, PA, USA), equipped with a flame-ionization detector and fused silica capillary column (BR2560 100 m x 0.25 mm internal diameter x 0.20 μm film thickness). The conditions of chromatography are described in Francisco *et al*. (2015).

4.2.3.2.3. Lipid oxidation

Lipid oxidation in meat was assessed through the quantification of thiobarbituric acid reactive substances (TBARS), following the method described by Grau *et al*., (2000). Briefly, 2 g of meat were homogenized in 1 ml of 0.3% aqueous ethylenediaminetetraacetic acid disodium salt (EDTA), 8 ml of 5% aqueous trichloroacetic acid and 5mL of 0.8% butylated hydroxytoluene (BHT) in hexane using an Ultra-Turrax T25 digital homogenizer (IKAWerbe GmbH & Co. KG, Staufen, Germany) for 30 sec at 19 000 rpm. The homogenates were centrifuged during 5 min at 1400*g* and the top hexane layer discarded. The bottom layer was filtered, and trichloroacetic acid was added to filtrate (5% aqueous) to make up a volume of 10 ml. Aliquot of 2.5 ml from the bottom layer was mixed with 1.5 ml of 0.8% aqueous 2-thiobarbituric acid and incubated at 70°C for 30 min. Following incubation, the mixture was

cooled under tap water and the absorbance measured at 532 nm in a double-beam UV-Vis scanning spectrophotometer (Helios alpha spectrophotometer, Thermo Scientific, Bremen, Germany). The 1,1,3,3 tetraethoxypropane standard curve was used for calculating the TBARS concentration and the results were expressed as mg of malonaldehyde (MDA)/kg of meat.

4.2.4. Trained sensory panel analysis

A trained sensory panel from Fonte Boa-INIAV, composed by eleven members, evaluated the meat sensorial characteristics in six sessions. For each session, were randomly selected 5 or 6 frozen loin joints, that were allowed to thaw for 24 h at 2 °C. After being deboned, the *Longissimus lumborum* muscles were cooked in an electric oven at 170 °C, until the internal temperature of meat reach 70 °C, which was monitored by an internal thermocouple (Thermometer, Eomega RDXL4SD, Manchester, USA). Each *Longissimus lumborum* sample was then trimmed of any external connective tissue and cut into 1×1×1 cm samples that were maintained in heated plaques at 60 °C, until tasting. The meat attributes under evaluation were odour, off-odour, tenderness, juiciness, flavour, off-flavour, and overall acceptability. For sensory analysis was applied a structured scale from 1 to 8, where the extreme values correspond: 1 - extremely soft (odour, off-odour, flavour, off-flavour), extremely tough (tenderness), extremely dry (juiciness) or extremely inacceptable (overall acceptability); 8 - extremely intense (odour, off-odour, flavour, off-flavour), extremely tender (tenderness), extremely juicy (juiciness) or extremely acceptable (overall acceptability).

4.2.5. Statistical analysis

Data were analysed as a completely randomized experimental design following a 2×2 factorial treatment arrangement using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The pen was considered the experimental unit and the animals with the pen as subsampling assuming a compound symmetry covariance structure. The homogeneity of variances was tested for a level of $P = 0.01$, and when significant, the models were fitted following the procedures described by Milliken and Johnson (2009).The main effects and their interaction were included in the model, and the interaction was removed, when not significant. Because most of the interactions were not significant, the results presented in the tables are the least square means obtained for each level of the main factors in study (*C. ladanifer* and DCP). A footnote was included when the interactions were significant. Standard error of the means is also presented. The level of statistical significance was set at $P< 0.05$. The occurrence of rumen mucosa parakeratosis was analysed similarly but using the PROC

GLIMMIX (SAS Institute Inc., Cary, NC), considering the binary distribution and the logit as link function.

Average daily weight gain (ADG) was determined individually by linear regression. The results of live slaughter weight and hot carcass weight were adjusted to initial live weight, being the results of carcass composition adjusted to hot carcass weight. Dry matter intake (DMI) was analyzed considering the observations from each week as a repeated measurement within pens, assuming a heterogeneous first-order autoregressive covariance matrix. Sensorial attributes were analyzed considering the observation from each panellist as the repeated measurement.

4.3. Results

4.3.1. Growth performance and carcass traits

Treatments did not affect the growth rate of lambs (Table 4.2) and the ADG was 288 ± 48.6g. The DMI increased 11.2% with inclusion of *C. ladanifer* in diets (*P* = 0.008), and tended to increase in diets with DCP as energy source (*P* = 0.066). Feed conversion ratio was not affected by the treatments, averaging 4.4 ± 0.24.

Table 4.2 - Effects of dietary inclusion of *Cistus ladanifer* (CL) (0 *vs* 15 % DM) and of Dehydrated citrus pulp (DCP) (Cereals *vs* DCP) on growth performance, carcass quality and chump and shoulder composition of Merino Branco lambs.

	Cistus ladanifer		**Cereals *vs* DCP**		**SEM**	***P* value**	
	0	**15**	**Cereals**	**DCP**		**Cistus**	**DCP**
Slaughter weight (kg)	30.8	30.6	30.4	31.0	0.54	0.750	0.445
DMI (g/d/kg $wt^{0.75}$)	120.8	134.3	120.8	134.4	3.85	0.008	0.066
ADG (g/d)	296.4	280.9	284.2	293.0	11.66	0.364	0.605
Feed/gain ratio	4.32	4.48	4.49	4.31	0.080	0.186	0.134
Probability of rumen parakeratosis (%)	0.67	0.27	0.33	0.60	0.180	0.181	0.370
Hot carcass weight (kg)	15.4	15.1	15.3	15.2	0.24	0.533	0.721
Cold carcass weight (kg)	15.0	14.7	14.9	14.8	0.25	0.565	0.709
Dressing (%) [ŧ]	49.7	49.5	50.3	48.9	0.30	0.510	0.003
Carcass conformation	2.61	2.24	2.38	2.48	0.168	0.148	0.669
Fat cover	2.74	2.70	2.75	2.69	0.166	0.893	0.803
HPJ (%)	53.9	54.6	54.4	54.1	0.36	0.200	0.620
Muscle (%)	58.4	58.4	57.9	58.9	0.68	0.999	0.318
Bone (%)	17.5	18.2	17.6	18.2	0.28	0.126	0.128
Muscle/Bone ratio	3.35	3.23	3.32	3.27	0.065	0.237	0.583
Subcutaneous fat (%)	22.7	21.9	23.1	21.4	0.70	0.430	0.105
Intermuscular fat (%)	11.3	10.2	11.2	10.3	0.40	0.068	0.161
KKCF (%)	3.14	3.06	3.09	3.12	0.198	0.776	0.929

[ŧ] – interaction CLxDCP, *P* =0.041; HPJ – higher price joints

Rumen mucosa parakeratosis was detected in 15 lambs but in a low degree of intensity (average score of 0.69 ± 0.559). Neither the general probability of occurrence (Table 4.2) nor the probability of occurrence of each score (Figure 4.1) were affected by treatments.

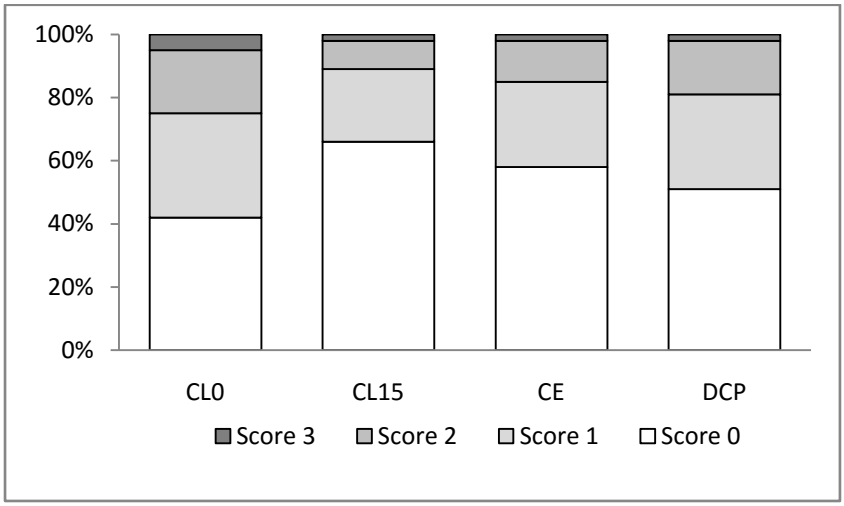

Figure 4.1 – Effects of *C. ladanifer* (CL0 *vs* CL15) and Dehydrated Citrus Pulp (DCP) inclusion (CE *vs* DCP) on the probability of occurrence of rumen mucosa parakeratosis in Merino branco lambs, according to the severity of the lesions

Hot and cold carcass weights were not influenced by treatments and presented average values of 15.2 ± 2.21 kg and 14.8 ± 2.18 kg, respectively (Table 4.2). For dressing percentage a significant interaction between *C. ladanifer* and DCP inclusion was found (P = 0.041). Cistus enhanced the depressive effect of DCP on dressing percentage. Without *C. ladanifer*, DCP reduced dressing percentage in 0.96 percentual points but when associated with *C. ladanifer*, the reduction was of 4.39 percentual points.

Treatments did not influence carcasses conformation and fat cover scores (data not shown). Four carcasses weighed less than 13 kg and were classified according to EU grid for carcasses of light lambs, being included in the “C” category and quality 1. The carcasses weighing more than 13 kg were graded with the SEUROP grid. For conformation, most of the carcasses (60.7%) were graded in class as O and 35.7% as R (good). For fat cover, most of the carcasses (64.3%) were graded in class as 3 (average) and 32.1% as class 2 (slight).

The replacement of cereals by DCP did not influence carcass composition (Table 4.2). However, the intermuscular fat tended to decrease with *C. ladanifer* inclusion in diets (P = 0.068).

4.3.2. Meat quality parameters

Physical attributes of lamb meat are presented in Table 4.3. Muscle pH and cooking loss were not affected by treatments and averaged 5.62 ± 0.038 and 22.3 ± 5.16%, respectively. Shear force was affected by *C. ladanifer* inclusion (P = 0.048), increasing 25.6%. Meat colour parameters were not influenced by treatments and the average values were 38.3 ± 2.09 for L^*, 13.9 ± 1.34 for a^*, 3.5 ± 1.34 for b^*, 13.9 ± 4.38 for H* and 14.4 ± 1.54 for C*. Moreover, the diets did not influenced the values of the indexes that represent the variation of meat colour during the seven days of storage, ΔE (7-0), ΔH* (7-0) and ΔC*, which averaged 6.5 ± 2.01, 14.5 ± 6.07 and -1.4 ± 2.4, respectively. Also, the lipid oxidation of meat after 7 days of storage was not affected by dietary treatments, averaging 1.34 ± 0.361 mg MDA/kg of meat.

Table 4.3– Effects of dietary inclusion of *Cistus ladanifer* (Cistus) (0 *vs* 15 % DM) and of Dehydrated citrus pulp (DCP) (Cereals *vs* DCP) on physical and chemical meat characteristics of Merino Branco lambs.

	Cistus ladanifer		**Cereals *vs* DCP**		**SEM**	***P* value**	
	0	**15**	**Cereals**	**DCP**		**Cistus**	**DCP**
Meat pH	5.63	5.62	5.62	5.63	0.010	0.894	0.964
Cooking losses (%)	21.3	23.4	22.7	21.9	1.72	0.404	0.771
Shear force (kgF)	3.91[a]	4.91[b]	4.54	4.28	0.326	0.048	0.582
Color parameters							
L^*[a]	37.8	38.8	37.6	39.0	0.52	0.206	0.085
a^*[a]	14.4	13.5	14.0	13.8	0.33	0.086	0.652
b^*[a]	3.69	3.34	3.36	3.67	0.358	0.493	0.551
Chroma[1]	14.8	13.9	13.4	14.3	0.39	0.119	0.872
Hue angle[1]	14.1	13.8	13.4	14.5	1.18	0.855	0.530
$\Delta E_{(7-0)}$	7.06	5.95	6.49	6.51	0.675	0.266	0.982
$\Delta Hue_{(7-0)}$	15.2	13.9	13.6	15.4	1.98	0.646	0.538
$\Delta Chroma_{(7-0)}$	-2.03	-0.74	-1.46	-1.31	0.696	0.214	0.881
TBARS (mg MDA/kg of meat)[2]	1.35	1.33	1.35	1.33	0.141	0.887	0.933

[1] – at day 0 of storage;[2] – at day 7 of storage; TBARS – Thiobarbituric acid reactive substances; MDA – malonaldehyde

Sensorial evaluation of lamb meat is presented in Table 4.4. The sensory panel considered the meat as tender (6.0 ± 1.29), with moderate initial and persistent juiciness (4.8 ± 1.30 and 5.1 ± 1.37 respectively), soft odour (3.2 ± 1.65) and soft flavour (3.4 ± 1.82). Some attributes were influenced by the dietary treatments. Tenderness was lower in meats from lambs fed with *C. ladanifer* (less 14%, P < 0.001) and fed with DCP (less 5%, P = 0.009). Initial juiciness was also depressed 16% by *C. ladanifer* (P < 0.001) and 5% by DCP (P = 0.029), but persistent juiciness only was significantly affected by the inclusion of *C. ladanifer* in the

diet (-15%, $P < 0.001$). Meat overall acceptability averaged 5.9 ± 1.15 and it was only affected by *C. ladanifer* inclusion, which reduced it in 10% ($P < 0.001$).

Table 4.4– Effects of dietary inclusion of *Cistus ladanifer* (Cistus) (0 *vs* 15 % DM) and of Dehydrated citrus pulp (DCP) (Cereals *vs* DCP) on sensorial meat characteristics of Merino Branco lambs.

	Cistus ladanifer		**Cereals *vs* DCP**		**SEM**	***P* value**	
	0	**15**	**Cereals**	**DCP**		**Cistus**	**DCP**
Trained sensory evaluation							
Tenderness	6.39^{b}	5.48^{a}	6.09^{b}	5.78^{a}	0.095	<.0001	0.009
Initial juiciness	5.16^{b}	4.33^{a}	4.87^{b}	4.62^{a}	0.093	<.0001	0.029
Persistent juiciness	5.47^{b}	4.64^{a}	5.16	4.95	0.101	<.0001	0.082
Odor intensity	3.15	3.30	3.24	3.21	0.110	0.294	0.841
Off-odor intensity	0.83	0.77	0.75	0.86	0.086	0.575	0.293
Flavor intensity	3.26	3.40	3.33	3.33	0.111	0.270	0.987
Off-flavor intensity	0.55	0.70	0.67	0.57	0.079	0.134	0.291
Overall acceptability	6.28^{b}	5.63^{a}	5.97	5.94	0.086	<.0001	0.724

4.3.3. Nutritional value of intramuscular fat

In Table 4.5 we present the content on intramuscular fat (IMF) and relevant nutritional FA and FA sums that were based on the individual FA composition (results not shown) of LT muscle and reported to 100 g of fresh meat. It was also considered the relative contribution of total fat and the fatty acid sums to energy content of fresh meat (112.1 kcal/100g).

None of the treatments influenced intramuscular fat content of meat which averaged 3.63 ± 0.723%. *C. ladanifer* reduced the total content of TFA in meat ($P = 0.016$) and their contribution to the energy content of the serving dose ($P = 0.015$). Replacing cereals by DCP increased the content of meat in 18:3 n-3 ($P = 0.048$).

Table 4.5– Effects of dietary inclusion of *Cistus ladanifer* (Cistus) (0 *vs* 15 % DM) and of Dehydrated citrus pulp (DCP) (Cereals *vs* DCP) on pulp (CP)) on nutritional value of fat Merino Branco Lambs, reported to 100 g of fresh meat.

		Cistus ladanifer		**Cereals *vs* DCP**		**SEM**	***P* value**	
		0	**15**	**Cereal**	**DCP**		**Cistus**	**DCP**
Nutrient (RDI [a])								
IMF (20-35% E[b])	mg/100g	3.86	3.40	3.52	3.74	0.201	0.134	0.470
	%TE[c]	30.4	27.5	28.4	29.4	1.16	0.101	0.566
SFA (<10% E[b])	mg/100g	1000	1020	993	1028	66.7	0.844	0.730
	%TE[c]	9.1	8.2	8.0	9.3	0.65	0.348	0.171
TFA (<1% E[b])	mg/100g	326[b]	254[a]	267	313	18.1	0.016	0.098
	%TE[c]	2.6[b]	2.0[a]	2.1	2.5	0.14	0.015	0.096
PUFA (6 – 11% E[b])	mg/100g	284	303	293	294	10.6	0.221	0.919
	%TE[c]	2.3	2.4	2.3	2.4	0.08	0.220	0.904
n-3 PUFA (0.5-2%E)	mg/100g	36.3	39.6	36.0	39.9	1.34	0.101	0.066
	%TE[c]	0.29	0.32	0.29	0.32	0.011	0.083	0.061
18:3*n*-3 (>0.5% E[b])	mg/100g	19.2	19.7	17.3[a]	21.6[b]	1.39	0.799	0.048
	%TE[c]	0.15	0.16	0.14	0.17	0.012	0.823	0.053
n-6 PUFA (2.5 - 9%E[b])	mg/100g	248	264	257	255	9.7	0.270	0.871
	%TE[c]	2.0	2.1	2.1	2.0	0.08	0.278	0.870
18:2*n*-6 (>2.5% E[b])	mg/100g	205	216	211	210	8.1	0.328	0.950
	%TE[c]	1.6	1.7	1.7	1.7	0.06	0.321	0.963
EPA+ DHA (>250mg/d)	mg/100g	9.01	9.81	9.22	9.60	0.693	0.430	0.730
CLA 9*c*,11*t*-18:2 (*Not established)*	mg/100g	45.8	34.4	36.1	44.1	5.51	0.171	0.326

[a] -RDI- Recommended dietary intake for adults (FAO, 2010); [b] –total energy of the diet ; [c] – average total energy of 100 g of fresh lamb meat, 112.1 kcal; IMF - intramuscular fat; FA - fatty acids; SFA- saturated fatty acids; TFA - *trans* fatty acids; PUFA - sum of *n*-6 + *n*-3 polyunsaturated fatty acids; n-6 PUFA - sum of *n*-6 PUFA (18:2n-6+C18:3n-6+20:2n-6+20:4n-6+22:4n-6); n-3 PUFA - sum of n-3 PUFA (18:3n-3+20:5n-3+22:5n-3+22:6n-3); EPA+DHA - sum of long chain n-3 PUFA (20:5n-3+22:6n-3).

4.4. Discussion

4.4.1. Growth performance and carcass traits

Lambs showed growth rates similar to those observed in previous trials with MB lambs fed with similar diets (Santos-Silva & Vaz-Portugal, 2001; Bessa *et al*., 2005; Francisco *et al*., 2015). However, the average value of 288 ± 48.6 g/d, is below the growth potential of MB breed, obtained when lambs were raised in intensive production systems (Santos-Silva & Vaz-Portugal, 2000; Santos-Silva *et al*., 2002b), which can be justified by the higher forage : concentrate ingredient ratio in the pelleted diets used in the present trial.

The inclusion of 150 g/kg DM of *C. ladanifer* had no effect on lamb's growth rate confirming the previous results refered by Jerónimo *et al.* (2010b), with a level of *C. ladanifer* inclusion

of 250 g/kg DM, and by Francisco *et al*. (2015), with a level of *C. ladanifer* inclusion of 50, 100 and 200 g/kg DM. High levels of dietary inclusion of CT may reduce feed intake (Rochfort *et al*., 2008). So, it could be expected that the inclusion of a tanniferous shrub like *C. ladanifer* in lamb diets, could cause a depression intake, in line with the results of Priolo *et al*. (2000). However, in the present trial the effect was opposite and intake increased about 11%.

In previous experiments, the inclusion of *C. ladanifer* in diets had no effects on lamb's dry matter intake (Jerónimo *et al*., 2010b; Francisco *et al*., 2015). Justifying the incoherence's in this set of results, Waghorn (2008) refers that the effect of CT in ruminant diets may be beneficial or detrimental on growth performances, depending of the level of inclusion and of the chemical structure.

Most of the carcasses were graded in class O for conformation and class 3 for fat cover of the SEUROP system, corresponding to carcasses with moderate muscularity and an average fat cover. In the present experiment, *C. ladanifer* inclusion in lamb's diet did not influence carcass fat cover score, what it was validated by data from dissection results of chump and shoulder. Our previous experiments suggested that the dietary *C. ladanifer* inclusion could increase carcass fat proportion (Jerónimo *et al.,* 2010b and Francisco *et al*., 2015). This response to *C. ladanifer* was not fully understood but one possible explanation could be an excess of energy supply relative to nitrogen enhanced by the reduction of protein absorption due to the effect of *C. ladanifer* tannins (Dentinho *et al.,* 2014). However, in the present experiment the effect of *C. ladanifer* in chump and shoulder fat proportion was not significant, indicating that the putative adipogenic effect of *C. ladanifer* is not consistent. In previous experiments the increase of *C. ladanifer* in diets was associated to an increase in fat content of diets and, consequently, of their energetic density. However, in the present experiment, all the diets had similar fat (± 80 g/kg DM) and crude protein (±170 g/kg DM) contents and less condensed tannins (4.55 mg/kgDM), which could be enough to prevent the expression of the adipogenic effect of *C. ladanifer*.

The effect of total replacement of cereals by DCP in lamb's growth performance was residual. Growth rate and feed conversion ratio were unaffected and DMI tended do increase (P=0.066). These results are supported by those of Bueno *et al*. (2004) and Caparra *et al*. (2007), but not by Rodrigues *et al*. (2008a), that observed a depression in DMI and ADG caused by the replacement of maize by DCP above 33% in Santa Inês lambs diet. Differences in palatability related in fruit composition of DCP and/or in the diets used in the different trials may justify the differences in the results.

Dressing percentage decreased when cereals were replaced by DCP, what can probably be explained by the differences in DMI. Although the interaction has not been significant (P =

0.525), DMI was higher for DCP15 diet (145 g/kgwt$^{0.75}$) than for the other diets, suggesting a higher weigh of gut and of digestive content, which justifies the lower value of dressing percentage that was observed.

Parakeratosis of rumen mucosa is a frequent pathologic condition in animals fed with high-grain low-forage diets, characterized by an excessive proliferation and abnormal cellular differentiation within the rumen epithelium (Steele, Greenwood, Croom, & McBride, 2012), that originates an excessively keratinized epithelium with reduced capacity for volatile FA absorption. Rumen parakeratosis has been associated with high incorporation of DCP in low forage diets (Pascual & Carmona, 1980; Bampidis & Robinson, 2006). In the present trial, diets included 50% of forage ingredients (lucerne or lucerne plus *C. ladanifer*) which probably explain the irrelevance on rumen parakeratosis of the replacement of cereals by DCP.

4.4.2. Meat quality parameters

It is described in several experiments that incorporating plants rich in tannins in lamb diets increases meat lightness (Priolo *et al*., 2000; Lanza *et al*., 2001; Priolo *et al*., 2005). In the present trial, in accordance to the results of previous trials of our research team (Jerónimo *et al*., 2012; Francisco *et al*., 2015), meat colour parameters were consistently not affected by the addition of Cistus in lamb diets, suggesting that the effects of dietary tannin sources on meat lightness might be dependent of the type of CT source.

The oxidative stability of colour and lipids during meat storage was not affected by *C. ladanifer*. The effect of cistus inclusion in lamb diets on meat oxidative stability has been previously explored by our research team, that reported the improvement of the oxidative stability of lipids (Jerónimo *et al*., 2012) and of colour and lipids (Francisco *et al*., 2015) in meat from oil supplemented and unsupplemented lambs. However, in this experiment the level of CT in the diets was lower than in the previous ones. Furthermore, the differences in the FA composition of lipid supplements among experiments may also be relevant. In both Jerónimo *et al.* (2012) and Francisco *et al*. (2015) it was used as lipid supplement a blend of 2 parts of linseed oil (source of 18:3 n-3) and 1 part of sunflower or soybean oil (sources of 18:2 n-6), respectively. However, in the present experiment it was exclusively used soybean oil, which caused a lower deposition of 18:3 n-3. The n-3 PUFA have higher susceptibility to oxidation than other PUFA with lower number of double bonds, namely 18:2 n-6 (Morrissey *et al*., 1998). We may hypothesize that, in the present trial, the balance between the effects, on meat oxidation, of the pro-oxidative agents, including the n-3 PUFA, and the antioxidant compounds of *C. ladanifer,* including CT, did not allow that the previously observed positive effect of dietary inclusion of *C. ladanifer* in improving meat stability was evidenciate.

Meat shear force increased 1 kgF due to *C. ladanifer* inclusion, result that was corroborated by the sensorial analysis, where meat of lambs fed with cistus showed a reduction of 14% of tenderness score. Previous observations from our team concluded that *C. ladanifer* inclusion in lamb's diet has no effect on meat shear force (Francisco *et al*., 2015) and, as far as we know, there are no reports referring significant effects of the inclusion of tanniniferous plants in lamb diets on meat tenderness.

In previous trials, cistus inclusion in the diet had no effect in overall acceptability of lamb meat (Jerónimo *et al*., 2012; Francisco *et al*., 2015). The present trial suggest that *C. ladanifer* can have a negative impact on meat acceptability by consumers, as a consequence of a reduction on tenderness ($r = 0.69$, $P < 0.001$), persistent juiciness ($r = 0.63$, $P < 0.001$) and initial juiciness ($r = 0.60$, $P < 0.001$) (data not shown) scores.Only in few studies it was explored the effect of dietary citrus by-products on meat colour, and the results also have been inconsistent. In agreement with our results, Rodrigues *et al*. (2008b) did not observed effect of the replacement of cereals by DCP on colour coordinates of lamb meat. Moreover, in pigs, the addition of ensiled citrus pulp did not influence the colour of meat (Cerisuelo *et al*., 2010). However, other authors reported a consistent reduction of *a** and C* in meat from lambs fed diets supplemented with DCP (Caparra *et al*., 2007; Inserra *et al*., 2014).

In disagreement to our results, Inserra *et al*. (2014) reported a positive effect of dietary DCP on colour and lipid oxidation of lamb meat after 3 and 6 days of storage, that it was attributed to the increase of phenolic compounds in the diets. Although the levels of phenolic compounds in DCP diets from Inserra et al. (2004) were lower than those observed in our experiment, the trial was longer and the lambs were slaughtered with heavier weights. These differences in methodology could affect meat lipid composition and also the susceptibility to oxidation, explaining the divergence between results of the two experiments.

The replacement of cereals by DCP had no effect on meat shear force, in accordance with Lanza *et al*. (2001), Scerra *et al*. (2001) or Caparra *et al.* (2007). However, the sensory panel scored better the tenderness of meat from lambs fed with cereals, according to Lanza et al. (2001).

4.4.3. Nutritional value of intramuscular fat

Average values for IMF in all treatments were below 5%, which is the reference value for a meat to be considered as a low fat food according to FAC (1990).

The energy content of 100 g of lamb meat in the present experiment ranged between 102.4 and 128.2 kcal. Based on fatty acid composition of intramuscular fat that will be presented in

a companion paper, we have evaluated how the results comply with the guidelines of FAO for Fat and Fatty Acids in Human Nutrition (FAO, 2010).

In what concerns to total fat and saturated fat, lamb meat fully comply the recommendations. However for *trans* fatty acids (TFA), the average value of their contribution to the total energy content of 100 g of meat, was estimated 2.3 ± 0.73 %, what is above the upper limit of 1% referred by FAO (2010). However, about 50% of that value (1.2 ± 0.66 %) was from vaccenic acid (11*t*-18:1). The biological effects of TFA are probably isomer-specific (Scollan *et al*, 2006) and the FAO recommendations (2010) did not take into account that point. Several authors claimed that, in terms of the effects on human health, the TFA should be considered separately according to its origin and to their isomer-specific health effects (Gebauer *et al*., 2011; Mapiye *et al.*, 2015). Vaccenic acid is the main product of the biohydrogenation of PUFA in high-forage fed ruminants (Scollan et al., 2014) and it should be considered as a "healthy fatty acid" (Mapiye *et al*., 2015), since it is the major precursor for the endogenous synthesis of CLA 9*c*,11*t*-18:2 isomer (rumenic acid) (Palmquist, 1994). Anti-cancerinogenic, anti-obesity, anti-diabetogenic, anti-atherogenic and anti-inflammatory effects are some of the health benefits attributed to rumenic acid (Mele *et al*., 2013; Wang & Proctor, 2013), but in 2010, the recommendations of FAO concerning dietary fatty acids intake did not include any reference to any CLA isomers. However, several authors suggest that the increase in 11*t*-18:1 and in 9*c*,11*t*-18:2 should be a main goal to improve the nutritional value of fat from ruminants (Mapiye *et al*., 2015). In the present experiment the intake of 11*t*-18:1 and 9*c*,11*t*-18:2 in a serving dose of 100 g of lamb meat ranged between 125 and 166 and between 34 to 46 mg, respectively. Daley et al. (2010) referred that the estimated values for daily intake of 9*c*,11*t*-18:2 needed to be effective in cancer prevention, are widely dispersed (3000, 620 e 95 mg/day for men). So it is essential to clarify this last aspect to evaluate the effective contribution of lamb lean meat to meet to the daily needs of 9*c*,11*t*-18:2 and also to consider the contribution of 11*t*-18:1, which it is converted in human muscle, to 9*c*,11*t*-18:2 by Δ-9 desaturase with an efficiency of about 30% (Mapiye *et al*., 2015).

The contribution of n-6 or n-3 PUFA to total energy in meat is below the minimums recommended by FAO (2010), in spite of the diets being supplemented with 6% soybean oil, which is an effective approach to improve nutritional value of meat, increasing n-6 PUFA and decreasing SFA content (Sinclair, 2007). The replacement of cereals by DCP increased the content in meat of the essential fatty acid linolenic (18:3 n-3) which is consistent with the results of Rodrigues et al. (2010). Dehydrated citrus pulp fat is richer in 18:3 n-3 than cereals (Rodrigues *et al*., 2010), which justify the results.

In the present experiment, the average amount of EPA plus DHA in a serving dose of lamb meat was 9.40 ± 2.49 mg, corresponding to 3.8% of the recommended daily intake for adults (250 mg/d) recommended by the FAO (2010).

4.5. Conclusions

With the results obtained in the present study, we confirmed the potential of dehydrated *Cistus ladanifer* leaves and soft stems to be incorporated in the forage fraction of complete diets for fattening lambs. In a diet with forage:concentrate ratio of 1:1, 15% inclusion of *Cistus ladanifer* had no negative impact on animal productivity or carcass composition, but the oxidative stability of meat was not improved. Meat acceptability was impaired due to the negative influence of *C. ladanifer* in meat tenderness and juiciness. The inclusion of *C. ladanifer* in the diets reduced the contribution of TFA to total energy of meat, in line with the nutritional recommendations of FAO. According to our results, there is no indication that the replacement of cereals by dehydrated citrus pulp can reduce the productivity of Merino Branco lambs during fattening or impair the quality of carcasses and meat. Furthermore, DCP improved the meat nutritional value due to the increase of 18:3 n-3.

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The authors declare not having a conflict of interest.

CHAPTER 5

Effect of replacing cereals by citrus pulp and of *Cistus ladanifer*, L. inclusion on oil supplemented diets for lambs on meat fatty acids

Effect of replacing cereals by citrus pulp and of *Cistus ladanifer*, L. inclusion on oil supplemented diets for lambs on meat fatty acids

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Artigo em preparação

Contribution of Alexandra E. Francisco to this article:

Alexandra E. Francisco participated in the manufacture of the diets, in the animal experiment and in the sample collection. Performed the data processing and statistical analysis, interpretation and discussion of the results, as well as in the writing of the manuscript.

ABSTRACT

The effects of the inclusion of *Cistus ladanifer*, L. (*C. ladanifer*) and dehydrated citrus pulp (DCP) in diets for growing lambs were evaluated on fatty acid composition and expression of desaturase genes on meat. Thirty two ram lambs were randomly assigned to 16 groups of 2 lambs each and 4 diets, resulting from the combination of the two factors (CL 0 *vs* 150 g/kg DM, and Cereals *vs* DCP). The inclusion of *C. ladanifer* in diets increased the proportions of 17:0, 19:1, 20:0, 12*c*-18:1, 16*c*-18:1 and 12*c*, 15*c*- 18:2 and DCP increased the proportions of 15:0, 17:0, 20:3 *n*-9/ 9c,11t,15c-18:3, 6*t*-/7*t*-/8*t*- 18:1, 12*t*-18:1, 12*c*-/18:1, 9*c*,13*t*-/8*t*,12*c*-18:2 and 12*c*,15*c*-18:2. The CLAs 11*t*,13*t*-18:2, 11*t*,13*c*-18:2, 10*t*,12*t*-18:2 also increased in muscle with DCP. Neither 11*t*-18:1 nor CLA 9c,11*t*-18:2 levels were influenced by the treatments. When combined with cereals, *Cistus ladanifer* inclusion in diets decreased the proportions of 10*t*,15*c*-/ 11*t*,15*c*- 18:2, CLA 10*t*,12*c*- 18:2, total 18:1 isomers, total biohydrogenation intermediates (BI), 10*t*-BI and *trans* FA (TFA), but not with DCP. In diets without *C. ladanifer*, the replacement of cereals by DCP resulted in a reduction of CLA 10*t*,12*c*- 18:2, and 10*t*-BI in muscle. A reduction of in the ratio n-6 : n-3 was obtained when DCP replaced cereals in the diets (P = 0.024). Dried citrus pulp slightly improved meat nutritional value, being interesting as cereal replacer in lamb diets.

Key words: lamb; dehydrated citrus pulp*; Cistus ladanifer;* fatty acid composition; meat; biohydrogenation intermediates

5.1. Introduction

The ruminant products are the main natural sources in human diet of ruminal biohydrogenation (RBH) intermediates vaccenic (11*t*-18:1) and rumenic (9*c*,11*t*-CLA) acids, which are currently considered as bioactive FA with beneficial effects in human health (Gebauer *et al*., 2011; Wang & Proctor, 2013). Enhancing their proportions in ruminant edible fats are important goals when strategies are being defined to improve the nutritional value of ruminants meat and milk (Mapiye *et al*., 2012). Anti-cancerinogenic, anti-obesity, anti-diabetogenic, anti-atherogenic and anti-inflammatory effects are some of the health benefits attributed to the rumenic acid (Bhattacharya *et al*., 2006; Mele *et al*., 2013; Wang & Proctor, 2013). Vaccenic acid is the predominant *trans* monoene isomer present in rumen and in the edible fat of ruminants fed with fibrous diets (Wang & Proctor, 2013; Scollan *et al*., 2014). In ruminants and humans, about 20% to 30% of the 11*t*-18:1 absorbed is converted in 9*c*,11*t*-CLA in tissues (Turpeinen *et al.*, 2002; Bhattacharya *et al*., 2006; Gruffat *et al*., 2008). Therefore, 11*t*-18:1 increment might be an important goal to the improvement of ruminant

nutritional value of edible fat. Moreover, the direct potential effect of 11*t*-18:1 in the protection against cardiovascular diseases (CVD) by is having increased attention and is being under debate and intensive research (Gebauer *et al.*, 2011; Tardy *et al*., 2011; Wang & Proctor, 2013; Gayet-Boyer *et al,* 2014).

The use of vegetable oils rich in PUFA, namely those with higher content in linoleic acid (18:2 n-6), in the diets of forage fed ruminants allows a higher deposition of rumenic and vaccenic acids in meat (Santos-Silva *et al*., 2004; Bessa *et al*., 2007; Jerónimo *et al*., 2009; Jerónimo *et al*., 2010). However, this strategy is dependent of basal diet and meat from ruminants fed with concentrate-rich diets, even when supplemented with PUFA-rich oils, present low proportions of 11*t*-18:1 and 9*c*,11*t*-CLA (Bessa *et al*., 2005; Francisco *et al*., 2015). The production of vaccenic acid is positively infuenced by a ruminal pH of 6.0 or above (Khanal & Dhiman, 2004) and starchy diets lead to low rumen pH. In such conditions the RBH pattern changes, and the predominant *trans* monoene in rumen and in meat fat, becomes 10*t*-18:1 over 11*t*-18:1 (10*t*-18:1 shift) (Bessa *et al*., 2005, Alves *et al*., 2013, Bessa *et al*. 2015). This has a negative impact in meat nutritional value as 10*t*-18:1, contrarily to vaccenic acid, is currently considered as prejudicial for human health (Wang *et al.*, 2012; Aldai *et al*., 2013).

Ruminants raised for meat production are normally fed with high energy diets, rich in cereals. In such conditions, the challenge is to maintain the high energy level of the diets, allowing high growth rates and sufficient carcass and intramuscular fat synthesis, and avoiding the 10*t*-18:1 shift in order to obtain edible products with high levels of vaccenic and rumenic acids. We have hypothesized that the replacement of cereals by other raw materials could combine those two goals, namely using by-products that cause a drastic reduction in starch intake. Citrus pulp is an agro-industrial by-product of the juice industry that is widely available for the industry of compound feeds for ruminants as dehydrated citrus pulp pellets (DCP). The main characteristics of DCP are a low starch and protein content and a high level of pectins and sugars that are readily and highly fermentable in the rumen (Bampidis & Robinson, 2006).The replacement of starch by pectins as the main source of energy for microbes prevents low rumen pH and favours the activity of cellulolytic bacteria and fibre degradability (Ben-Ghedalia *et al*., 1989). This would preserve the normal biohydrogenation pathways, and led to an increase of 11*t*-18:1 and 9*c*,11*t*-CLA in meat when animal are supplemented with a vegetable oil. Recently, our team demonstrated this concept in the milk FA of dairy ewes (Santos-Silva *et al*., 2016) but it never was tested in ruminants for meat purpose. Due to the high digestibility of the fibrous components, the replacement of cereals by DCP in small ruminant diets do not compromise animal performance (Bhattacharya & Harb, 1973; Bueno *et al*., 2004; Bampidis & Robinson, 2006; Rodrigues *et al*., 2008a;) or

meat quality (Lanza *et al.*, 2001; Scerra *et al*., 2001; Caparra *et al.,* 2007; Rodrigues *et al*., 2008b).

Cistus ladanifer is an aromatic shrub frequent in the Mediterranean ecosystems, with high levels of plant secondary metabolites, such as condensed tannins (CT) (Dentinho *et al*., 2005). It can be included up to 10% in lamb diets where forage represented 50% DM (Francisco *et al*., 2015) or even more (25% DM) in all forage diets (Jerónimo *et al*., 2010b, 2012), without negative effects in growth performances, carcass and meat quality traits. Jerónimo *et al*. (2010b) observed that *C. ladanifer* exerted a synergic effect with vegetable oil on the pattern of RBH isomers present in meat, enhancing the deposition of vaccenic and rumenic acids. Therefore, the main objective of the present trial was to evaluate if the replacement of cereals by DCP, and the combination of DCP and *C. ladanifer* may promote the enrichment of vaccenic and rumenic acids in meat from lambs fed with diets supplemented with soybean oil.

5.2. Materials and methods

5.2.1. Animals and management

The animal handling followed EU Directive 2010/63/UE concerning animal care. Thirty-two Merino Branco (MB) ram lambs born in autumn 2013 were reared with dams on extensive grazing until weaning at approximately 60 days of age. At weaning, lambs were transported to the Unidade Estratégica de Investigação e Serviços em Produção e Saúde Animal (UEISPA- INIAV), at the Polo de Investigação da Fonte Boa, Instituto Nacional de Investigação Agrária e Veterinária (Fonte Boa-INIAV), located at Vale de Santarém, Portugal, where the trial was performed. Lambs were housed and randomly assigned to 16 pens; 2 lambs per pen and 4 pens per treatment, according to a completely randomized experimental design with a 2×2 factorial arrangement of treatments. The first factor was the inclusion of *C. ladanifer* in the diets (0 and 150 g/kg DM) and the second factor was the inclusion of DCP in the diets, replacing cereals (cereals *versus* DCP). According to this, the experimental diets were: 1) cereals; 2) cereals with 15% of *C. ladanifer*; 3) DCP and; 4) DCP with 15% of *C. ladanifer*. All diets included 60 g/kg of soybean oil. Leaves and soft stems of *C. ladanifer* shrubs were harvested in Portugal (39°30'36"N/8°19'00''W) in April 2013, dried at room temperature, cut in small pieces, and milled to a final particle size of 3 mm. Diets were prepared and milled in the UEISPA-INIAV. The ingredient composition of the four diets and the chemical composition, obtained as the average of the results of three pooled samples of each diet, are presented in Table 4.1.

Animal management during the experimental period, slaughter and sample collection procedures are described on Chapter 4.

5.2.3. Analytical procedures

Analytical procedures concerning to feed and meat lipid composition, except for CLA, are described on Chapter 4.

5.2.3.1. CLA composition of meat lipids

For the analysis of the CLA composition in meat, the methyl esters of CLA isomers were individually separated by triple silver-ion columns in series, using a high performance liquid chromatography system equipped with auto-sampler and diode array detector (DAD) adjusted to 233 nm, according to the procedure previously reported by Alfaia *et al*. (2006). The quantification of the individual CLA isomers in meat, was obtained with a combination of gas chromatography and 3 Ag^{+}-HPLC methods, as described in Bessa *et al.* (2007).

5.2.3.3. Gene expression

Total RNA was isolated from lamb muscle samples by using QIazol reagent and further purified using the RNeasy mini kit with on column DNase I treatment based on the manufacturer's protocol (all from Qiagen, Hilden, Germany). The RNA quantification, evaluation of quality and subsequent analysis for determination of gene expression levels by quantitative PCR were performed according to the methodology descripted in Francisco et al. (2016) submitted to Animal.

5.2.4. Statistical analysis

Data were analysed as a completely randomized experimental design following a 2×2 factorial treatment arrangement using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The pen was considered the experimental unit and the animals within the pen as subsamples, assuming a compound symmetry covariance structure. The homogeneity of variances was tested for a level of $P = 0.01$, and when significant, the models were fitted following the procedures described by Milliken & Johnson (2009). The main effects and their interaction were included in the model.

The results of meat fatty acid composition were adjusted to intramuscular fat content and when necessary, Pearson correlations among variables were conducted. The level of statistical significance was set at $P < 0.05$.

5.3. Results

5.3.1. Lipid composition of *Longissimus thoracis* muscle

Both intramuscular fat (IMF) and total of fatty acid (FA) content of meat were not significantly influenced by the diets and averaged, respectively, 3.6 ± 0.72% and 2666 ± 601.9 mg/100g of fresh muscle (Table 5.1), but *C. ladanifer* tended to decrease ($P = 0.053$) IMF when fed jointly with DCP but not with cereals.

The FA composition of LT muscle is presented in Tables 5.1 and 5.2. The major FA present was *c*9-18:1, followed by 16:0 and 18:0. None of the proportions of these FA was influenced by the diets and averaged 28.1 ± 2.96%, 21.3 ± 1.46% and 15.6 ± 1.70% respectively. In spite of presenting fairly high levels, neither 11*t*-18:1 nor CLA 9*c*,11*t*-18:2 levels, were influenced by the treatments, averaging 53.6 ± 27.35 and 14.7 ± 6.75 mg/g FA, respectively. The inclusion of *C. ladanifer* in diets increased the proportions of 17:0, 19:1, 20:0, 12*c*-18:1, 16*c*-18:1 and 12*c*,15*c*-18:2. The inclusion of DCP increased the proportions of 15:0, 17:0, 20:3 *n*-9/9c,11t,15c-18:3, 6*t*-/7*t*-/8*t*- 18:1, 12*t*-18:1, 12*c*-/18:1, 9*c*,13*t*-/8*t*,12*c*- 18:2 and 12*c*,15*c*-18:2. Also the CLAs 11*t*,13*t*-18:2, 11*t*,13*c*-18:2, 10*t*,12*t*-18:2 increased in muscle with the dietary replacement of cereals by DCP. A reduction of 12.3% in the ratio between n-6 FA and n-3 FA was obtained with DCP inclusion in the diets ($P = 0.024$). Significant interactions between *C. ladanifer* and DCP inclusion were observed for 10*t*,15*c*-/11*t*,15*c*-18:2, the CLAs 12*t*,14*t*-18:2 and 10*t*,12*c*-18:2, the total of 18:1 isomers, the total of 18:2 non-conjugated isomers, the total of biohydrogenation intermediates (BI), 10*t*-BI and TFA (Table 5.2). For CLA 12*t*,14*t*-18:2 and the total of 18:2 non-conjugated isomers, their proportions in muscle were increased by the combination of *C. ladanifer* and DCP. For 10*t*,15*c*-/ 11*t*,15*c*-18:2, CLA 10*t*,12*c*-18:2, total 18:1 isomers, total BI, 10*t*-BI and TFA, *C. ladanifer* inclusion in diets decreased the proportions of these FA in muscle when combined with cereals, but not with DCP. Moreover, in diets without *C. ladanifer*, DCP reduced CLA 10*t*,12*c*-18:2, and 10*t*-BI in muscle. Also for 10*t*-18:1, its proportion was reduced when *C. ladanifer* was added to the diet with cereals (36.0 *vs*. 10.7 mg/g FA) and, in diets without *C. ladanifer*, when cereals were replaced by DCP (36.0 *vs*. 11.1 mg/g FA). Those observations did not reach significance ($P = 0.069$), because of the high variability within lambs in the same diet, especially in diets with cereals (Table 5.2). The ratio 10*t*-18:1 / 11*t*-18:1 and the stearoyl-CoA desaturase activity index (SCD17i) computed as 9*c*-17:1 / 9*c*-17:1+17:0, were not affected by the dietary treatments ($P > 0.05$) averaging, respectively, 0.58 ± 0.939 and 0.30 ± 0.093.

Table 5.1 –Effects of *Cistus ladanifer* (CL) (0 *vs* 15 % DM) and Dehydrated citrus pulp (DCP) inclusion (Cereals *vs* DCP) on intramuscular fat (IMF, g/100g meat), fatty acid (FA) content (mg/100 g meat) composition (mg/g of total FA) of total FA of Merino Branco lamb meat

	0% *Cistus ladanifer*		**15% *Cistus ladanifer***		**SEM**	***P* value**		
	Cereals	**DCP**	**Cereals**	**DCP**		**CL**	**DCP**	**CL×DCP**
IMF	3.48	4.23	3.57	3.24	0.252	0.096	0.418	0.053
Total FA	2424	3150	2563	2523	203.8.	0.325	0.177	0.139
FA composition								
12:0	0.95	1.06	0.82	0.92	0.171	0.458	0.555	0.979
14:0	21.1	20.0	19.3	21.7	1.58	0.965	0.690	0.313
i-15:0	0.94	0.97	0.71	0.93	0.127	0.305	0.339	0.466
a-15:0	1.09	1.16	1.07	1.27	0.151	0.780	0.402	0.692
9*c*-14:1	0.84	0.66	0.61	0.77	0.160	0.747	0.957	0.324
15:0	2.22	2.48	2.13	2.64	0.165	0.851	0.036	0.480
i-16:0	0.93	1.07	0.81	0.94	0.110	0.286	0.239	0.941
16:0	219	212	209	214	6.0	0.501	0.819	0.331
9*c*-16:1	13.1 (1.20)	11.6 (0.63)	12.1 (0.56)	11.1 (0.40)	-	0.346	0.141	0.745
9*t*-16:1	0.76	0.74	0.89	0.79	0.253	0.726	0.822	0.882
i-17:0	2.92	4.16	2.58	2.92	0.398	0.076	0.061	0.299
7*c*-16:1	1.62	2.12	1.75	1.96	0.193	0.936	0.087	0.486
a-17:0	0.94	0.82	0.97	1.22	0.110	0.082	0.552	0.124
17:0	6.96	6.99	7.22	7.71	0.258	0.003	0.011	0.331
i-18:0	0.80	0.68	0.86	0.83	0.210	0.958	0.938	0.550
9*c*-17:1	3.30	2.58	3.35	3.17	0.640	0.632	0.489	0.683
18:0	144[a]	164[b]	162[b]	154[ab]	5.8	0.535	0.134	0.035
9*c*-18:1	274[ab]	284[ab]	300[b]	268[a]	8.95	0.592	0.244	0.050
18:2n-6	86.0	74.0	83.7	83.8	5.34	0.510	0.274	0.300
19:1	0.82	0.70	0.98	0.97	0.082	0.023	0.434	0.508
20:0	1.05	0.95	1.44	1.66	0.146	0.003	0.686	0.293
18:3*n*-6	0.68	0.75	0.89	0.95	0.129	0.146	0.620	0.994
11*c*-20:1	0.86	0.81	0.77	0.79	0.140	0.684	0.895	0.822
18:3*n*-3	6.55	7.36	7.36	8.07	0.432	0.110	0.094	0.918
20:2*n*-6	1.10 (0.313)	0.66 (0.117)	0.68 (0.106)	0.82 (0.111)	-	0.528	0.432	0.170
20:3*n*-9[1]	1.50	1.85	1.61	1.74	0.074	0.956	0.006	0.201
22:0	1.76	1.61	1.84	1.94	0.129	0.149	0.870	0.370
20:4*n*-6	14.0	15.2	15.9	13.8	0.75	0.766	0.525	0.063
20:5*n*-3	2.31	2.10	2.24	2.38	0.184	0.586	0.858	0.391
22:4*n*-6	1.71	1.32	1.50	1.29	0.226	0.615	0.210	0.696
22:5*n*-3	3.81	4.02	3.94	3.98	0.345	0.895	0.704	0.814
22:6*n*-3	1.41	1.37	1.47	1.62	0.302	0.617	0.852	0.775
Total BCFA	7.63	8.83	6.82	8.13	0.598	0.242	0.053	0.931
SFA	386	396	391	390	9.6	0.990	0.604	0.576
PUFA	119	110	119	119	6.53	0.551	0.463	0.560
n-3PUFA	14.1	14.8	15.1	16.1	0.86	0.222	0.355	0.846
n-3 LC PUFA	7.55	7.48	7.69	8.02	0.766	0.676	0.865	0.807
n-6 PUFA	105	95.7	104	102	6.02	0.638	0.356	0.545
n-6 LC PUFA	17.3	17.6	18.6	16.3	0.69	0.980	0.156	0.106
Others [2]	24.8	29.5	33.8	28.2	7.37	0.618	0.952	0.511
SFA/PUFA	0.31	0.26	0.31	0.32	0.076	0.272	0.426	0.158
n-6:*n*-3 ratio	7.7	6.3	6.9	6.5	0.34	0.453	0.024	0.204

[1]-coelutes with 9*c*,11*t*,15*c*- 18:3; [2]-Includes about 27.1 mg/g FA of dimethylacetals and 2.00 mg/g of undentified peaks; BCFA - Branched chain fatty acids (*i*-15:0+ *a*-15:0+ *i*-16:0+ *i*-17:0+ *a*-17:0+ *i*-18:0); SFA - saturated fatty acids; PUFA - sum of *n*-6 + *n*-3 polyunsaturated fatty acids; *n*-6 PUFA - sum of *n*-6 PUFA (18:2 n-6+C18:3 n-6+20:2 n-6+20:4 n-6+22:4 n-6); *n*-3 PUFA - sum of n-3 PUFA (18:3 n-3+20:5 n-3+22:5 n-3+22:6 n-3); n-6 LC-PUFA - sum of long chain n-6 PUFA (20:2 n-6+20:4 n-6+22:4 n-6); n-3 LC-PUFA - sum of long chain n-3 PUFA (20:5 n-3+22:5 n-3+22:6 n-3)

Table 5.2 – Effects of *Cistus ladanifer* (CL) (0 *vs* 15 % DM) and Dehydrated citrus pulp (DCP) inclusion (Cereals *vs* DCP) on biohydrogenation intermediates (BI) (mg/g FA) of Merino Branco lamb meat

	0% *Cistus ladanifer*		**15% *Cistus ladanifer***		**SEM**	***P* value**		
	Cereals	**DCP**	**Cereals**	**DCP**		**CL**	**DCP**	**CL×DCP**
18:1 isomers								
6*t*-/7*t*-/8*t*-	2.75	3.55	2.84	4.39	0.250	0.092	<0.001	0.180
9*t*-	4.03 (0.362)	3.90 (0.195)	3.59 (0.168)	4.32 (0.180)	-	0.969	0.244	0.119
10*t*-	36.0 (11.45)	11.1 (0.52)	10.7 (4.42)	16.1 (4.43)	-	0.183	0.197	0.069
11*t*-	54.7	64.7	41.8	53.4	11.41	0.318	0.350	0.943
11*c*-/15*t*-	12.6	11.5	11.1	11.9	0.57	0.396	0.739	0.148
12*t*-	6.56	7.17	6.02	8.58	0.542	0.444	0.011	0.111
12*c*-	9.00	9.80	13.7	20.2	1.38	<0.001	0.019	0.072
13*c*-	0.70	1.00	1.21	1.21	0.299	0.263	0.610	0.643
16*t*-/14*c*-	1.77	2.06	2.34	2.47	0.235	0.070	0.373	0.749
16*c*-	1.36	1.16	1.44	1.79	0.133	0.023	0.580	0.072
total	130^{b}	113^{ab}	95.0^{a}	126^{b}	8.97	0.257	0.435	0.024
18:2 isomers								
nonconjugated								
9*c*,13*t*-/8*t*,12*c*-	2.81	3.47	3.00	3.69	0.277	0.498	0.034	0.958
8*t*,13*c*-/9*c*,12*t*-	2.08	2.22	2.16	2.19	0.271	0.920	0.741	0.845
9*t*,12*c*-	0.96	0.71	0.65	0.88	0.112	0.563	0.942	0.066
11*t*,15*c*- /10*t*,15*c*-	2.82^{bc}	2.00^{ab}	1.66^{a}	3.08^{c}	0.281	0.894	0.306	0.002
12*c*,15*c*-	0.13	0.29	0.45	0.94	0.142	0.007	0.043	0.295
Total	8.82^{a}	8.66^{a}	7.92^{a}	10.8^{b}	0.542	0.280	0.026	0.020
conjugated								
12*t*,14*t*-	0.10^{a}	0.11^{a}	0.11^{a}	0.18^{b}	0.007	0.002	<0.001	0.027
11*t*,13*t*-	0.16	0.24	0.14	0.26	0.021	0.892	0.001	0.465
10*t*,12*t*-	0.13	0.20	0.11	0.18	0.017	0.310	0.003	0.819
9*t*,11*t*-	0.2	0.27	0.26	0.30	0.038	0.888	0.732	0.548
11t,13c-	0.31	0.48	0.28	0.49	0.067	0.916	0.019	0.811
10*t*,12*c*-	0.41^{b}	0.16^{a}	0.15^{a}	0.31^{ab}	0.064	0.440	0.541	0.018
9*c*,11*t*-	15.3	17.1	12.7	13.7	2.86	0.316	0.625	0.894
8*t*,10*c*-	0.24	0.28	0.18	0.18	0.047	0.110	0.746	0.668
7*t*,9*c*-	1.13	0.99	0.96	1.12	0.093	0.828	0.894	0.151
Total	18.3	20.1	15.1	17.0	3.02	0.324	0.543	0.985
Total C18:2 BI	27.2	28.8	23.0	27.8	2.75	0.380	0.254	0.593
Total BI	157^{b}	142^{ab}	118^{a}	154^{b}	11.0	0.255	0.358	0.046
10*t*-BI‡	37.0^{b} (11.45)	9.41^{a} (1.147)	11.2^{ab} (4.225)	17.9^{ab} (4.225)	-	0.241	0.172	0.048
TFA*	128^{b}	109^{ab}	86.1^{a}	113^{b}	8.34	0.045	0.611	0.024
***t*10/*t*11 ratio**	1.37 (0.816)	0.15 (0.015)	0.36 (0.181)	0.43 (0.181)	-	0.444	0.256	0.216
SCD17i[1]	0.34	0.26	0.30	0.29	0.043	0.887	0.266	0.422

‡ 10*t*-BI includes: 10*t*-18:1; 10*t*,2*t*-18:2 and 10*t*,12*c*-18:2; *TFA includes: 6*t*-/7*t*-/8*t*-18:1 + 9*t*-18:1+ 10*t*-18:1+ 11*t*-18:1 + 12*t*-18:1 + 11*c*-/15*t*-18:1 + 16*t*-/14c -18:1 + 9c,13t-/8t,12c-18:2 + 8t,13c-/9c,12t- 18:2 + 9t,12c-18:2 + 11t,15c- /10t,15c-; SCD 17i = (9*c*-17:1/(9*c*-17:1+17:0))

5.3.2. Desaturases gene expression

The results for the gene expression level of desaturases are presented in Table 5.3. *Cistus ladanifer* reduced Δ6-desaturase (*FADS2*) mRNA level (*P* = 0.034) and the same trend was observed for Δ5-desaturase gene (*FADS1*) (*P* = 0.060). The *SCD* mRNA level (Δ9-desaturase gene) was correlated with the amount of total meat lipids (r= - 0.42 *P* = 0.017), the percentage of 9*c*-14:1 (r= + 0.57 *P* = 0.001), 18:3 n-3 (r= - 0.42 *P* = 0.016) and 22:6 n-3 (r= + 0.41 *P* = 0.023), the SCD14i (9*c*-14:1/ 9*c*-14:1+14:0) (r= + 0.63 *P* < 0.0001), and 20:5 n-3/20:5 n-3+18:3 n-3 ratio (r= + 0.43 *P* = 0.014). It also tended to be positively correlated with SCD17i (r= + 0.34 *P* = 0.058). The Δ5-desaturase mRNA level was positively correlated with 10*t*-18:1 (r= + 0.36 *P* = 0.044), 10*t*-BI (r= + 0.36 *P* = 0.046), and *t*10/*t*11 ratio (r= + 0.42 *P* = 0.016). A trend of correlation between *FADS1* mRNA expression and SCD17i was also observed (r= + 0.34 *P* = 0.056). The *FADS1* expression correlated negatively with the percentage of 18:3n-3 in muscle (r= - 0.40 *P* = 0.022). The *FADS2* mRNA level only was correlated with *FADS1* mRNA level (r= + 0.71 *P* < 0.001) and with *SCD* mRNA level (r= + 0.36 *P* = 0.044). The mRNA levels of *FADS1* and *SCD* were also positively correlated to each other (r = + 0.36 *P* = 0.046).

Table 5.3- Effects of *Cistus ladanifer* (CL) (0 *vs* 15 % DM) and Dehydrated citrus pulp (DCP) inclusion (Cereals *vs* DCP) on relative mRNA level (arbitrary units x 100) of the codifying genes for Δ9-desaturase (*SCD*), Δ6-desaturase (*FADS2*) and Δ5-desaturase (*FADS1)* enzymes

	0% *Cistus ladanifer*		15% *Cistus ladanifer*		SEM	*P* value		
	Cereals	DCP	Cereals	DCP		CL	DCP	CL×DCP
SCD	65	43	33	66	15.2	0.760	0.736	0.095
FADS2	19	16	14	15	1.0	0.034	0.358	0.080
FADS1	96	69	64	67	8.2	0.060	0.173	0.105

5.4. Discussion

Obtaining ruminant meat enriched in 11*t*-18:1 and 9*c*,11*t*-18:2 is easily achieved using forage basal diets supplemented with vegetable oil containing C18 PUFA (Santos-Silva *et al.*, 2004; Bessa *et al.*, 2005). Moreover, in forage based diets, the inclusion of tanniferous forage sources, like *C. ladanifer*, can the positive amplify the effect of lipid supplementation by inhibiting the last step of biohydrogenation and increasing the availability of 11*t*-18:1 (Jerónimo *et al.*, 2010b). However, high forage basal diets, provide only a weak stimulus to lipogenesis compared to high concentrate diets, and thus the SCD, necessary for converting

the absorved 11*t*-18:1 into 9*c*11*t*-18:2 in the tissues, is not at its maximum activity (Bessa *et al*., 2015).

In common practice, ruminants intensively finished are currently fed with high energy diets, containing cereals as major ingredients, which promote high growth rates and lipogenic activity including SCD activity. However, in those conditions, the lipid supplementation is usually ineffective to increase 11*t*-18:1 and 9*c*11*t*-18:2 in meat due to the alterations of rumen biohydrogenation pathways that led to the production of 10*t*-18:1 in detriment of 11*t*-18:1 (*trans*-10 shift), as recently discussed by Bessa *et al*. (2015). Using, diets containing a medium amount of forage ingredients (i.e. 1:1 forage:concentrate ratio) as a basal diet, might be a good compromise between the extreme situations although the risk of the occurrence of the *trans*-10 shift is still high as demonstrated in previous experiment (Francisco *et al*. 2016, submitted to Animal). We hypothesized that replacing cereal component by DCP in these medium forage basal diets, would diminished the risk of the occurrence of the *trans*-10 shift while maintaining the upregulation of SCD, resulting in higher CLA deposition in muscle. Moreover, we also hypothesized that, after preventing the occurrence of the *trans*-10 shift and maintain a high SCD activity, the partial replacement of dietary forage component (i.e. lucerne) by *C. ladanifer* would exarcebate the rumen production and tissue availability of 11*t*-18:1 resulting in further increase of 9*c*11*t*-18:2 in meat.

The results showed that all treatments had high, but similar, levels of 11*t*-18:1 (≈5.4 % of total FA) and 9*c,*11*t*-18:2 (≈1.5 % of total FA) in meat. Thus, the anticipated outcome was not achieved and the hypothesized metabolic drives might not be strong enough to overcome other factors of the complex physiologic network that determine the final fatty acid composition of of meat.

The prevention of *trans*-10 shift and increasing availability of 11*t*-18:1 was our hypothesized starting point. In fact, it was clear that replacement of cereals in the diet reduced the individual variability regarding this trait (see Fig. 5.1 and standard errors in Table 5.2). Although it is clear that both high starch content favours the occurrence of the *trans*-10 shift in the rumen and that PUFA supplementation exarcebate that risk, it is also clear that a large individual susceptibility to the occurrence of the *trans*-10 shift is present (Rosa *et al*., 2014; Bessa *et al.*, 2015). The present results confirm the high variablility in animals fed with cereal diet and that DCP clearly reduce the risk of *trans*-10 shift occurrence.

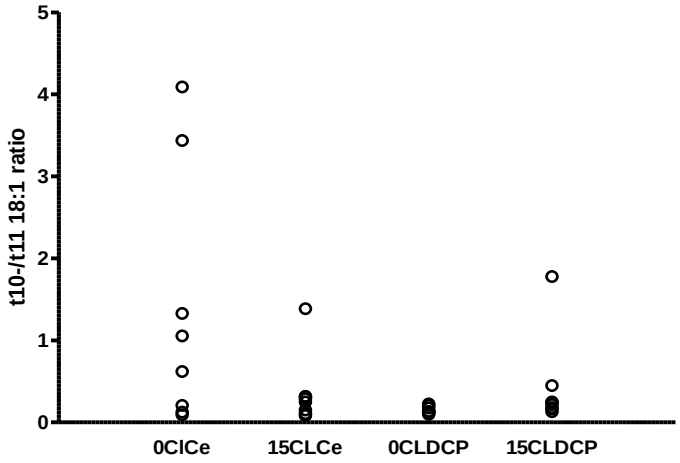

Figure 5.1- Individual variability regarding to 10*t*/11*t*- 18:1 ratio in the *Longissimus thoracis* muscle of lambs. Values higher than 1 correspond to the *trans*-10 shift occurrence. 0CLCe- Diet with 0% *C. ladanifer* and cereals; 15CLCe- Diet with 15% *C. ladanifer* and cereals; 0CLDCP- Diet with 0% *C. ladanifer* and dehydrated citrus pulp (DCP); 15CLDCP- Diet with 15% *C. ladanifer* and DCP.

In a previous study, the incorporation of *C. ladanifer* into high oil medium forage diets similar to those used in the present experiment, resulted in increasing risk of *trans*-10 shift occurrence (Francisco *et al*., 2016, submitted to Animal). However, in the present experiment *C. ladanifer* tended (interaction CLxDCP, $P = 0.069$) to reduce 10*t*-18:1 when incorporated in cereal diets. What was expected from the inclusion of *C. ladanifer* to diets was to promote the increase of the BI, mostly *trans*-18:1 isomers, due to the proposed effect of its condensed tannins on blocking the last step of the rumen biohydrogenation (i.e. reduction of *trans*-18:1 isomers to 18:0). However, this did not occur and *C. ladanifer* decreased the 18:1 BI and increased the 18:0 when included in cereal diets and had no effect when included in DCP diets. The reason for that results are not clear to us, but the fact that the *C. ladanifer* vegetative material used in the present experiment had lower condensed tannin content than in previous experiments (Jerónimo *et al*., 2010b and Francisco *et al*., 2015) plus the fact that the diets containing *C. ladanifer* resulted in lower C18 PUFA intake, might contribute to explain the lack of the expect effect.

Other objective was to confirm if we could obtain a lipogenic activity, particularly of Δ-9 desaturase with DCP diets similar to that which is achieved with cereals diets. The IMF tended to be higher in the diet containing DCP and 0% of cistus (CLxDCP interaction, $P = 0.053$). This suggests that DCP itself is able to replace cereals in diet, maintaining the energy flow and the stimulus to the lipid anabolic metabolism. This observation also confirms other reports where the IMF did not decrease when cereals in the diet were replaced by DCP (Rodrigues *et al.*, 2010; Lanza *et al.*, 2015). We evaluated the expression of mRNA of *SCD*

gene and the ∆-9 desaturase activity indirectly by product/substrate ratio. The *SCD* is the gene that codifies for Δ9-desaturase, the key enzyme for the synthesis in tissues of CLA 9*c*11*t*-18:2 from the desaturation of 11*t*-18:1. In this study, *SCD* mRNA level was positively correlated to SCD17i (r = 0.34 *P* = 0.058), which is considered as the best indirect descriptor for the activity of Δ9-desaturase in tissues (Bessa *et al*., 2015). Neither mRNA expression nor the Δ9-desaturase activity indices (SCD17i and SCD*t*11i) indicated changes in the SCD activity. This explains the fairly linear relationship between the 11*t*-18:1 and 9*c,*11*t*-18:2 in muscle (Fig. 5.2), which indicates that the efficiency of conversion of 11*t*-18:1 into 9*c,*11*t*-18:2 remained constant among diets and thus that should be the availability of 11*t*-18:1 that limited the 9*c,*11*t*-18:2 production.

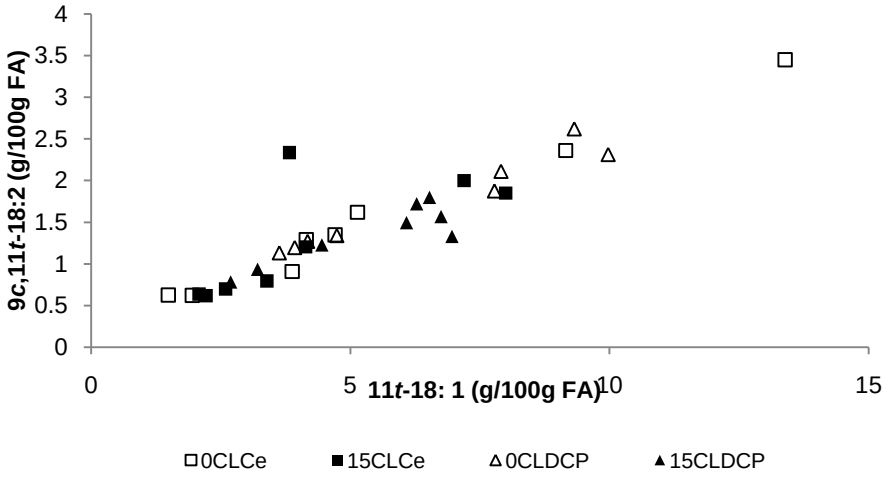

Figure 5.2- Relationship between 9*c*,11*t*-18:2 and 11*t*-18:1 concentration (g/100g fatty acids) in the *Longissimus thoracis* muscle of lambs. 0CLCe- Diet with 0% *C. ladanifer* and cereals; 15CLCe- Diet with 15% *C. ladanifer* and cereals; 0CLDCP- Diet with 0% *C. ladanifer* and dehydrated citrus pulp (DCP); 15CLDCP- Diet with 15% *C. ladanifer* and DCP.

In ruminant muscle, the very long chains PUFA (LC-PUFA) are located almost exclusively in membrane phospholipids and formed by elongation and desaturation (via Δ6- and Δ5-desaturases) of C18 PUFA (Alvarenga *et al*., 2015; Bessa *et al*., 2015). In a previous work we reported that the inclusion of *C. ladanifer* on lamb diets, depressed the n-3 LC-PUFA in muscle but, paradoxally it was found a weak positive correlation between condensed tannin intake and the expression of *FADS1* (Δ5-desaturase gene) and no effect on *FASD2* (Δ6-desaturase gene) expression (Francisco *et al*, 2016, submitted to Animal). In the present experiment, the inclusion of *C. ladanifer* in diets reduced the *FASD2* mRNA level and tended to reduce the *FASD1* mRNA level in muscle but these had no reflex on PUFA present in meat. Thus, no clear conclusions can be drawn about the putative direct effect of *C. ladanifer*

on the expression of desaturase genes and about its effects on LC-PUFA concentration in the lamb meat.

5.5. Conclusions

In all diets, the content of 11*t*-18:1 and 9*c*,11*t*-18:2 was high. The replacement of cereals by DCP in a medium forage diet (1:1 forage to concentrate ratio) supplemented with soybean oil, reduced the occurrence of the t*rans*-10 shift and maintained the SCD activity but did not resulted in increased concentration of 11*t*-18:1 and 9*c*,11*t*-18:2. The inclusion of *Cistus ladanifer* to these diets did not promoted the accumulation of biohydrogenation intermediates as expected.

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The authors declare not having a conflict of interest.

CHAPTER 6

General discussion

In the research reported on the present thesis we evaluated the effectiveness of three nutritional strategies to enhance the content in bioactive FA with beneficial effects to human health on meat from lambs reared in intensive production system. To accomplish this objective were performed two experiments: **Experiment 1 -** inclusion of increasing levels of *C. ladanifer* and of a vegetable oil blend in the diets of lambs (Chapter 2 and 3) and **Experiment 2-** inclusion of *Cistus ladanifer* L. and replacement of cereals by DCP, in high soybean oil diets of lambs (Chapter 4 and 5). Those experiments involved the nutritional strategies in study: a) dietary inclusion of *C. ladanifer,* with the intent of increase the proportions of BI on meat lipid composition; b) lipid supplementation of the diets, to provide lipid substrate to RBH and to increase PUFA deposition in meat fat and c) replacement of cereals by DCP in the diets, to manipulate ruminal conditions in order to reduce *trans*-10 shift and consequently increase the deposition of vaccenic (11*t*-18:1) and rumenic (9*c*,11*t*-18:2) acids in intramuscular fat.

In the present chapter we will discuss the effects of each individual strategy on the productive performance of lambs, meat organoleptic quality, including meat oxidative stability and evaluation by sensory panel, and their effectiveness to improve the fatty acid composition and nutritional quality of meat intramuscular fat. The contribution to the Dietary Reference Value (DRV) of some important FA and FA classes of a 100g serving dose of the meats obtained is also discussed.

6.1. Effects of dietary strategies on growth performances, carcass and meat quality

Dietary inclusion of Cistus ladanifer L.

Despite the beneficial effects of CT in ruminant diets, at high levels of dietary inclusion it may affect feed intake (Rochfort *et al.*, 2008) and growth performance of ruminants (Vasta *et al.*, 2008). In the two experiments, we confirmed that the incorporation up to 200g/kg of leaves and soft stems of *C. ladanifer* replacing an equivalent proportion of dehydrated lucerne, in isoenergetic and isonitrogeneous diets for growing lambs is well tolerated, without negative effects on DMI and growth performances. Even for the higher level of *C. ladanifer* (20%) associated to high levels of CT, (14.3 to 16.3 g/kg DM), the DMI was high, suggesting a good acceptability of lambs to those diets, except, perhaps, for the lambs which diets combined simultaneously high levels of *C. ladanifer* and oil suplementation. In that situation the variability of DMI and ADG was higher than in the others, suggesting that the tolerance of lambs to these diets was close to the upper limit. In the second experiment, with balanced diets for metabolic energy and protein, containing 6% of soybean oil, the incorporation of

15% DM of *C. ladanifer* in the diets (i.e. 30% of the forage fraction of diet and 3.53 to 5.58 g CT/kg DM), had no negative effects on lamb's growth performance. Thus, the results of the present study are in line to those obtained in 2010 by our research team concerning the potential use of *C. ladanifer* as alternative forage source in the diets of growing ruminants (Jerónimo *et al.*, 2010b). At that time, the level inclusion of *C. ladanifer* reached 250g/kg of DM in an all forage diet (Jerónimo *et al.*, 2010b).

It is also important that the alternative dietary strategies in study did not affect negatively the sensorial quality of meat.

Several experiments indicate that incorporating plants rich in tannins in lamb diets increases meat lightness (Priolo *et al.*, 1998; Priolo *et al.*, 2000; Lanza *et al.*, 2001; Priolo *et al.*, 2005). Our results, as it was previously observed by Jerónimo *et al.* (2012), show that meat colour parameters were not affected by the addition of *C. ladanifer* in lamb diets, suggesting that the effects of dietary tannin sources on meat lightness must depend on the type of CT source.

Oxidation of lipids is a major cause of deterioration in the quality of muscle foods and can directly affect other meat quality traits such as colour stability, texture, flavour, nutritive value and the safety of the food (Buckley *et al.*, 1995). Meat colour changes during storage are associated to alterations on myoglobin biochemistry namely with the increase of metmyoglobin production, causing the browning of meat and depreciating meat quality perceived by the consumers that typically associate bright red colour of meat with freshness (Mancini & Hunt, 2005). In recent years, plant secondary compounds such as CT were studied as potential natural antioxidants which may be included in ruminant diets through tanniferous feed sources, reducing meat lipid oxidation (Luciano *et al.*, 2011b; Jerónimo *et al.*, 2012) and improving colour stability (Priolo *et al.*, 2000; Luciano, *et al.*, 2009b). In the first experiment our results show that increasing levels of *C. ladanifer* inclusion in lamb's diet effectively prevent the meat colour changes and lipid oxidation during 7 days of refrigerated storage. However, in the second experiment the inclusion of 15% *C. ladanifer* on the diets did not influence meat colour parameters and lipid oxidative stability during the same 7 days of storage. Jerónimo *et al.* (2012) reported that the inclusion of 25% *C. ladanifer* in lamb's diet reduced meat lipid oxidation but no effect was observed for meat colour variation. In spite of all the experiences have used the same source of tannins (*C. ladanifer*), the level of CT in the diets of the second experiment of this research was lower than in previous ones and these difference may be the most plausible explanation for the lack of effect of *C. ladanifer* on both meat colour and lipid oxidative stability in second experiment. Several environmental factors may affect CT concentration in plant tissues (Guerreiro *et al.,* 2016). The two experiments used the same vegetal tissues (leaves and soft stems developed in the correspondent growing season) collected, at the same localization and at the same time of

the year (March-April), but in different years. Perhaps the reasons for such large variation in CT content of *C. ladanifer* might be due to climatic differences in each year. Other possible explanation for the divergent results might be the differences in the FA composition of lipid supplements used in the two experiments (i.e. an oil blend containing linseed oil *vs.* soybean oil) and hence the concentration of 18:3 n-3 in the meat and susceptability to oxidation. In the second experiment, where soybean oil was used as supplement, the content in 18:3 n-3 in meat remained low and thus meat was less prone to oxidation.

In both experiments, it was observed that the dietary *C. ladanifer* inclusion affected meat sensory quality and that can reduce overall acceptability by consumers. It was associated to a reduction on lamb meat tenderness juiciness and increase of meat shear force (only in the second experiment). The average value of meat shear force obtained with 15% *C. ladanifer* dietary inclusion in the second experiment was 4.9 ± 0.30 kgF, higher than that obtained in the first experiment for the maximum level of *C. ladanifer* (20%) (3.9 ± 0.40 kgF). Those values are also high when compared to those reported by Sañudo *et al.* (2003) from a survey on lamb's meat texture from different European production systems. *Cistus ladanifer* contains high levels and a high variety of polyphenolic compounds, as phenolic acids and several flavonoids other than CT (Barrajon-Catalan *et al.*, 2010; Guerreiro *et al*., 2016). In the second experiment, the influence of *C. ladanifer* on meat tenderness and juiciness was more expressive. Despite of the lower concentration of CT that was already referred total phenols content averaged similarlly to the level of 20% *C. ladanifer* dietary inclusion of the first experiment, or even higher for the diet which simultaneously included *C. ladanifer* and DCP. It has been recently reported that phenolic compounds can exert both antioxidant and pro-oxidant effects, depending of the chemical structure of the compound, its concentration in the muscle, the oxidative conditions and the free radical source (lipids *vs* proteins) (Estévez, 2011; Lund, Heinonen, Baron *&* Estevéz 2011; Utrera & Estevéz, 2013). Oxidation of muscular proteins has been linked to changes in protein solubility and functionality which negatively affects meat tenderness and juiciness (Lund *et al.*, 2011). The calpain system is the main responsible for the post-mortem tenderization of meat due to its proteolytic activity on myofibrilar proteins (Koohmaraie & Geesink, 2006). However, to be functional, the proteins of the calpain system must be in a reduced form (Lund *et al.*, 2011). If phenolic compounds oxidize the calpain system proteins, the result will be a decrease on the myofibrilar proteins proteolysis and thus, an impaired meat tenderization (Lund *et al.*, 2011). Moreover, phenolic compounds may form complex interactions with the oxidized myofibrillar proteins with further negative effects on meat tenderness (Estevéz *et al.*, 2011; Lund *et al.*, 2011). Since we did not assess the level of meat protein oxidation, we only may hypothesise that the negative effects of dietary *C. ladanifer* on meat tenderness might result from the pro-

oxidant action of some of the polyphenolic compounds other than CT, on the early *post-mortem* oxidative processes of muscle proteins.

The inclusion of alternative vegetable feed sources rich in tannins can increase the intensity of off-flavours in lamb meat, as it was observed by Whitney *et al.* (2011) when lamb diets were supplemented with redberry juniper. However, this effect is probably dependent of the type and level of the feed source used. Priolo *et al.* (1998) reported that the inclusion of carob pulp in lamb's diet was not detected in meat. Jerónimo *et al.* (2012) also reported that 25% inclusion of *C. ladanifer* in lamb diets had no effect on meat sensory properties despite the strong influence of dietary *C. ladanifer* on the meat volatiles profile (Vasta *et al.*, 2010b). The evaluation of meat sensory attributes of the first experiment suggested that *C. ladanifer* inclusion in the diets may increase lamb meat flavour and off-flavour intensities. Despite this, in both experiments, meats from lambs fed with *C. ladanifer* were considered as soft flavoured and with extremely soft intensity of off-flavours.

Vegetable oil supplementation

The effects of supplementing concentrate-based diets with vegetable oils on ruminant performance, carcass and meat quality, including meat lipid profile, have been reported by different research teams (Cooper *et al.*, 2004; Bessa *et al.*, 2005; Santé-Lhoutellier, Engel & Gatellier, 2008; Manso *et al.*, 2009; Radunz *et al.*, 2009). Moderate lipid supplementation of diets increases their energy density which may lead to a reduction of DMI (Palmquist, 1994) with negative consequences to animal performance. Scollan *et al.* (2006) referred as maximum level for lipid proportions in ruminant diets, 60 g/kg of DM. In the present study, the dietary supplementation with 80g/kg DM of a blend of linseed and soybean oil in the first experiment, corresponded to an average a fat in the diets of 111 g/kg of DM, which determined a significant reduction of DMI. However, the energy intake was similar and the lambs' productive performance was not affected, despite the ADG of lambs fed with diets supplemented with 80g/kg DM oil (267 ± 20.2 g) have been below the potential growth for Merino Branco breed. In the second experiment all diets were supplemented with 60 g/kg DM of soybean oil, corresponding to an average fat in diets of 80 g/kg DM. Growth rate was higher in the second experiment (288.4 ± 48.64 g *vs* 279 ± 19.8 g and 267 ±19.8 g, for 4 and 8% of oil supplementation) and feed:weight gain ratio was lower (4.4 ± 0.24 *vs* 5.2 ± 0.31 and 6.2 ± 0.31 for 4 and 8% of oil supplementation) were observed. As expected, in the first trial lipid supplementation slightly increase carcasses fatness and consequently decreased muscle, in accordance to the results of other experiments of our research team with MB lambs (Santos-Silva *et al.*, 2004; Bessa *et al.*, 2005).

The effects of dietary supplementation with lipids rich in PUFA on meat shelf-life, namely on myoglobin oxidation, colour degradation and lipid oxidative stability are subjects of concern for researchers, due to the high susceptibility of PUFA to oxidise, especially of n-3 PUFA (Morrissey *et al.*, 1998). Consumer purchasing choice is highly influenced by meat colour (Mancini and Hunt, 2005) and interactions between the products of PUFA oxidation and the heme group of myoglobin moiety may enhance myogloblin oxidation and subsequently, meat discoloration (Faustman *et al.*, 2010). After cooking, volatile compounds derived from lipid oxidation may compromise meat eating quality through the appearance of off-flavours such as rancid (Calkins & Hodgen, 2007; Nute *et al.*, 2007). In the first experiment, despite the increase of PUFA (12.5 % *vs* 15.7% *vs* 17.4% for 0, 4 and 8% of oil supplementation) and of n-3 PUFA (2.87 % *vs* 5.03% *vs* 5.92% for 0, 4 and 8% of oil supplementation) on meat lipids, colour deterioration and lipid oxidation of meat were not affected. However, in meat from lambs supplemented with 80g/kg DM the sensory evaluation by a tasters panel, identified a higher intensity of off-flavours, despite of being always very low scores (1.34 ± 0.113) and off-flavour intensity was positively correlated to n-3 PUFA content(r=0.34; P = 0.012) and to 18:3 n-3 proportion in intramuscular lipids (r=0.30; P = 0.032). Nevertheless, the variability of off-flavours identified by the pannelists was high (18 off-flavours, including rancid), suggesting that lipid oxidation was not the only contributor to the development of off-flavours, since it mainly contributes to the development of rancid flavour. Soldatou *et al.* (2009) concluded that, in lamb meat, rancidity could only be detected for TBARS values higher than 4.4 mgMDA/kg of meat. However, in the present study, rancid flavour was detected by panelists at TBARS levels as low as 1.4 to 1.6 mgMDA/kg, corresponding to the meat from lambs fed with diets supplemented with 40 and 80g/kg DM oil, respectively. These results showed that the thresholds for detection of off-flavours on meat are dependent of the experience and sensitivity of panelists. Supplementing the diets of lambs with 80g/kg DM oil blend resulted in a reduction on meat overall acceptability of 0.5 points when compared to meat from lambs fed without oil in diets. However, in the context of the final consumer perception, this difference may be probably inexpressive.

Replacement of cereals by dehydrated citrus pulp

The total replacement of cereal grains by DCP in the second experiment of this research had an inexpressive effect on intake and, productive performance as well as on carcass and meat quality of lambs. The absence of effect on ADG due to the replacement of cereals by DCP in a complete pelleted diet confirms the previous results obtained by other authors (Bueno *et al.*, 2004; Caparra *et al.*, 2007). The inclusion of DCP in lamb diets increased DMI, while other authors reported that the effect was null (Bueno *et al.*, 2004; Caparra *et al.*, 2007) or even opposite (Bhattacharya and Harb, 1973; Rodrigues *et al.* 2008a). Differences on

palatability of the different DCP sources may be the reason for different results amoung studies. The replacement of cereals by DCP had no effect in carcass fatness and composition, which is in accordance to Prado *et al.* (2000), Lanza *et al.* (2001) and Henrique *et al.* (2004).

Despite the reduction on lipid oxidation during 3 and 6 days of storage of meat from lambs fed with dietary DCP reported by Inserra *et al.* (2014), we did not observe any effect of DCP on meat lipid stability. Inserra *et al.* (2014) attributed the results to the high concentration of phenolic compounds in the diets with the inclusion of DCP. The levels of phenolic compounds in DCP diets from Inserra *et al.* (2014) were lower than those observed in our study. However differences in methodology may affect the quantity of phenolic compounds ingested and meat lipid composition, thus its susceptibility to oxidation, which may explain the divergence between results of the two trials. Moreover, the type and level of bioactive compounds on DCP depend on the citrus fruit composition which is quite variable (Bampidis & Robinson, 2006), what may also contribute to differences on effects of dietary inclusion of DCP on meat colour and lipid stability between studies. Concerning to meat sensory quality DCP reduced the scores for meat tenderness, which was also reported by Lanza *et al.* (2001). However, those effects were small and were not repercurted on meat global acceptability assessed by the sensory panel.

6.2. Effects of dietary strategies on lipid composition and nutritional value of intramuscular fat

In both experiments of the present research, meats were low fat since their IMF content was below the threshold of 5% established by FAC (1990) (averaging 2.1 and 3.6%, for experiment 1 and 2, respectively).

With the results of the first experiment, that involved the evaluation of the lipid profile of TAG and of phospholipids fractions of IMF, we confirmed that BI, including health beneficial 11*t*-18:1 and CLAs such as 9*c*,11*t*-18:2 are preferentially deposited in the TAG lipid fraction This represents a restraint to the design of strategies to promote these FA in meat, since it imply the simultaneous increase of their proportion on the total FA and of TAG and IMF content of meat, as discussed by Bessa *et al.* (2015). Since a high meat fat content is generally associate to a high SFA content and dietary and health recommendations discourage the consumption of SFA, the European consumers tend to depreciate meat higher IMF content (McNeill, 2014).

6.2.1. Effects on lipid composition of intramuscular fat

Dietary inclusion of Cistus ladanifer L.

Our results confirmed that the dietary inclusion of *C. ladanifer* combined with vegetable oil favours the accumulation of BI and modulates RBH pathways, modifying the FA composition and the nutritional value of lambs´ meat. The effects depend, not just of the content of phenolic compounds and CT of *C. ladanifer*, but also on the composition of the basal diet, including its content on UFA. Jerónimo *et al.* (2010b), in high-forage diets supplemented with 6% vegetable oil, reported that *C. ladanifer* increased the 11*t*-18:1 and 9*c*,11*t*-18:2, with no effect on 10*t*-18:1. On the present research, we used a basal diet with a forage:concentrate ratio of 1:1, and *C. ladanifer* increased total BI and 10*t*-18:1 on meat but not 11*t*-18:1 and 9*c*,11*t*-18:2. This particularly occurs when diets were supplemented with vegetable oil. In the first experiment, most of animals expressed *trans*-10 shift and its magnitude depended of both levels of *C. ladanifer* inclusion and oil supplementation. In the second experiment, the effect of Cistus on the content of 10*t*-18:1 and 10*t*-BI was inconsistent reducing those FA when the diet contains cereal grains, but not DCP. This incongruence between the results of the two experiments was probably related to the low CT content of *C. ladanifer* in the second trial. Moreover, the addition of *C. ladanifer* to DCP was not successful to reduce 10*t*-18:1 and 10*t*-BI and simultaneously increased 11*t*-18:1 and 9*c*,11*t*-18:2 in meat.In the second experiment we confirmed the high individual variability for 10*t*-BI deposition on IMF that was observed in the first experiment. This high individual variability indicates that diet is not the sole determinant of the *trans*-10 shift. Lambs from the same pen, fed with a same diet, showed quantitatively quite different responses to *trans*-10 shift occurrence, related to level of 10*t*-18:1 and other related BI. This individual susceptibility to the *trans*-10 shift needs to be better understood and to be considered in the design of experiments aiming to study dietary strategies to reduce the risk of occurrence of *trans*-10 shift.

In the first experiment, we detected a slight reduction of n-3 LC PUFA on lamb meat fed with diets including *C. ladanifer*. However, this was not confirmed in the second experiment. Also, inconsistent results were observed for the effect of *C. ladanifer* on the expression of *FADS1* (Δ5-desaturase) FADS2 (∆-6 desaturase) genes.

Vegetable oil supplementation

We confirmed that the lipid supplementation of diets is an efficient strategy to manipulate the lipid profile of ruminant meats. Supplementing lamb diets with 50% concentrate with a vegetable oil blend composed by dietary sources of 18:2 n-6 and 18:3 n-3 (experiment 1) increased BI, PUFA, including n-3 PUFA, and reduced SFA levels on meat FA. However,

and due to the prevalence of *trans*-10 shift, lipid supplementation was mainly associated to the extensive increase of the 10*t*-18:1 proportion and not of 11*t*-18:1. Furthermore, the combination of oil supplementation with *C.ladanifer* inclusion, promoted the meat content of 10*t*-18:1.

The oil blend used in experiment 1, contained 2 parts of linseed oil (rich in 18:3 n-3) and 1 part of soybean oil (rich in 18:2 n-6). This oil blend effectively increased 18:3 n-3 but not the the n-3 LC-PUFA content in meat. Similar results were reported by Albertí *et al.*,(2014) and Bessa *et al.*(2007) and can be explained by the low efficiency of conversion of 18:3 n-3 into its long-chain derivatives FA (Scollan *et al.*, 2001; Sinclair, 2007). Moreover, it was reported that *trans*FA can affect LC-PUFA synthesis and incorporation in cellular membrane phospholipids though the inhibition of Δ-5 and Δ-6 desaturase activities as competitive inhibitors (Mahfouz, 1981; Kinsella *et al.*, 1981). In the experiment 1, the gene expression of Δ-5 and Δ-6 desaturases did not correlate with the level of *trans* FA present in the phospholipids. However, the 20:5 n-3/(18:3 n-3+20:5 n-3) and the 22:5 n-3/(18:3 n-3+22:5 n-3) ratios expressing the activity of desaturase and elongase of n-3 PUFA were highly and negatively correlated to *trans* FA (r=-0.35, *P*=0.016 and r=-0.39, *P*=0.006, respectively). Furthermore, lipid supplementation of diets with oils rich in n-3 and n-6 PUFA can reduce Δ-5 and Δ-6 genes expression, as it was referred by Nakamura *et al.*(2002) and confirmed by Herdmann *et al.* (2010). Our results are not in line with those two authors, but the levels of both 18:2 n-6 and 18:3 n-3 in meat correlated negatively with 20:5 n-3/(18:3 n-3+20:5 n-3) and the 22:5 n-3/(18:3 n-3+22:5 n-3) ratio (r=-0.41, *P*=0.004 and r=-0.43, *P*=0.002, respectively).

It is clear that in absence of *trans*-10 shift, the supplementation of diets with 18:2 n-6 rich sources such as soybean oil, is an efficient strategy to increase CLA in the muscle of ruminants (Santos-Silva *et al.*, 2004; Bessa *et al.*, 2005; Jerónimo *et al.*, 2009). In this set of experiments we have used mixed diets, with equal proportions of concentrate and forage. In these type of diets the results of lipid supplementation are difficult to predict and present a high individual variability. The results of the second experiment showed that it is possible to increase 11*t*-18:1 and 9*c*,11*t*-18:2 by the inclusion of lipids in ruminants fed with mixed diets, but future research is needed to fully unsderstand the metabolic processes linked with the biohydrogenation of dietary FA in rumen.

Replacement of cereals by dehydrated citrus pulp

Dried citrus pulp is low in starch, but it is rich in pectin and soluble sugars (Rihani, 1991) and it has a high content in digestible fibre (pectin) promoting a fermentative pattern more close to forages than starchy feedstuffs (Ariza *et al.*, 2001). Thus it is expected that it can generate

a rumen environment favorable to the RHB pathways leading to the synthesis of 11*t*-18:1 and 9*c*,11*t*-18:2 (Zened, *et al.*, 2013), In the second experiment, we hypothesised that the replacement of cereals by DCP on a diet containing 50% of forage and supplemented with soybean oil could increase 11*t*-18:1 and 9*c*,11*t*-18:2 in lamb meat by preventing the occurrence of the *trans*-10 shift. However, we were not able to confirm statistically this hypothesis because the effect ofcereal containing diets was not consistent on inducing the *trans*-10 shift in lambs. Moreover, the number of experimental units was fairly low considering the large individual variability.

When DCP replaced cereals in the diet without Cistus, the deposition of 10*t*-18:1 was reduced ($P<0.10$) from 3.6 % to 1.1 % (- 69.0%) and of 10*t*-BI from 3.7 % to 0.9% (-74.6%) which is consistent with our hypothesis. However, those differences were not clearly statistically significant, due to the high individual variability observed.

Due to the effects of *C. ladanifer* on increasing the BI obtained in the first experiment, we expected that the simultaneous dietary incorporation of *C. ladanifer* and DCP would results in a larger increase of 11*t*-18:1 and 9*c*,11*t*-18:2. However, in experiment 2, the incorporation of *C. ladanifer* into the DCP diets, did not result in higher proportions of those FA in muscle lipids.

6.2.2. Effects on nutritional value of meat intramuscular fat

According to FAO (2010) the evaluation of the nutritional value of fats for human consumption must be based on total fat content and on FA composition, including SFA, *trans*FA, n-6 and n-3 PUFA, specifically 18:2 n-6, 18:3 n-3 and EPA+DHA. Dietary recommendations for the intake of those FA are stablished on the basis of the percentage of the Energy intake (%E) and, those values, for adults, are presented in Table 6.1. The energy requirement of an adult averaged 2000 kcal (EFSA,2013) and the energetic value of fats is 9 kcal/g.Considering the lipid content of LT muscle, was possible to determine the Dietary Reference Value (DRV) for the relevant FA and FA classes reported by FAO (2010) and to quantify the potential contribution (%) to the DRV of fat, recommended FA and FA classes, of a 100g serving dose of the lamb meats fed with the different diets.

Table 6.1– Recommended values for the dietary intake of total fat and of fatty acids for healthy adults by FAO (2010).

Total fat, FA or FA classes	Dietary recommendations of daily intake for normal adults according to FAO (2010)
Total Fat	20-35%E[a] (400-700kcal)[b]
SFA	<10%E (200kcal)
Trans MUFA	<1%E (<20kcal)
PUFA	6 – 11%E (120 – 220kcal)
n-3 PUFA	0.5-2%E (10-40 kcal)
18:3*n*-3	>0.5%E (>10kcal)
EPA+DHA	>250 mg/day
n-6 PUFA	2.5 - 9%E (50-180kcal)
18:2*n*-6	>2.5 %E (>50kcal)

[a] - Percentage of total energy intake.[b] – For an average energy requirement of 2000 kcal (EFSA, 2013).

Table 6.2 presents the average content of intramuscular fat and of some individual FA and FA classes with nutritional importance of lamb meat of both the experiments reported in this thesis and the percentual contribute to the DRV.

Table 6.2 - Average values of intramuscular fat content of meat (IMF) (g/100g meat), of content in 100g of meat (mg /100g meat) of 18:0, 11*t*-18:1, 10*t*-18:1, 9*c*,11*t*-18:2, 18:3 n-3 and 18:2 n-6 and of SFA, TFA, *cis*MUFA, PUFA, n-3 PUFA, n-6 PUFA and EPA+ DHA obtained in the different dietary strategies studied in the present thesis. For FA and FA classes that have daily recommendations for their intake, the contribution (%) of a serving dose of 100g lamb meat to the Dietary Reference Value (DRV*), of each FA or FA classes is presented. For *C. ladanifer* inclusion and vegetable oil supplementation strategies, average values that result from the first experiment (for, 5% CL, 10% CL and 20% CL and 0% Oil blend, 4% Oil blend and 8% Oil blend), that were significantly different (P<0.05), are presented in separated columnus.

		***C. ladanifer* dietary inclusion**				**Vegetable oil supplementation**				**Replacing cereals by dehydrated citrus pulp**	
		5% CL	**10% CL**	**20% CL**	**15% CL**	**0% Oil blend**	**4% Oil blend**	**8% Oil blend**	**6% soybean oil**	**Cereals**	**DCP**
IMF			2.1		3.6		2.1		3.6	3.5	4.2
	%DRV		2.7 - 4.7%		4.7 – 8.2%		2.7 - 4.7%		4.6 – 8.2%	4.5 – 7.9%	4.8 - 9.4%
SFA			524		1020		524		1076	954	1308
	%DRV		2.4%		4.6%		2.4%		4.8%	4.3%	5.9%
18:0			172		404		172		417	349	510
TFA		46.7	61.8	91.5	254	42.6	70.4	87.0	290	310	341
	%DRV	2.1%	2.8%	4.1%	11.4%	1.9%	3.2%	3.9%	13.1%	14.0%	15.1%
11*t*-18:1			21.2		125		21.2		145	136	195
10*t*-18:1			36.0		34.9	10.6	39.5	58.0	46.0	86.5	27.7
9*c*,11*t*-18:2			9.3		34.4		9.3		40.1	37.8	53.6
***cis*MUFA**			434		810		433		847	730	1039
PUFA			189		303		189		294	289	277
	%DRV		0.8 - 1.4%		1.2 – 2.3%		0.8 - 1.4%		1.2 – 2.2%	1.2 – 2.2%	1.1 - 2.1%
***n*-3 PUFA**			57.2		39.6	37.8	60.9	72.7	37.9	34.4	38.0
	%DRV		1.3 – 5.2%		0.90 – 3.6%	0.9 – 3.4%	1.4 – 5.5%	1.6 - 6.5%	0.86 – 3.4%	0.78 – 3.1%	0.86 - 3.4%
18:3*n*-3			33.7		19.7	15.1	36.5	49.5	19.5	15.9	22.5
	%DRV		3.1%		1.8%	1.4%	3.3%	4.5%	1.8%	1.4%	2.0%
EPA+DHA			11.7		9.8		11.7		9.4	9.2	8.7
	%DRV		4.7%		3.9%		4.7%		3.8%	3.7%	3.5%
***n*-6 PUFA**			131		264		131		256	254	239
	%DRV		0.7 – 2.4%		1.3 – 4.7%		0.7 – 2.4%		1.3 – 4.6%	1.3 – 4.6%	1.2 - 3.6%
18:2*n*-6			103		216		103		211	211	199
	%DRV		1.8%		3.9%		1.8%		3.8%	3.8%	3.6%

*- The DRV for each FA and FA sum was determined based on the dietary recommendations for fat intake of FAO (2010) (Table 6.1) and on an average energy requirement for adults of 2000 kcal recommended by EFSA (2013)

Some of the effects of *C. ladanifer* on FA proportions did not repercute on the content on meat of those FA, as it was the case of 10*t*-18:1 and of the sum of EPA plus DHA. However, meat from lambs of the first experiment fed with 20% *C. ladanifer* presented a higher TFA content than that from lambs fed 5 and 10% *C. ladanifer*. Despite the high content of TFA on the meat from the second trial (254 mg/100 g meat) which represented about 11% of the DRV for that group of FA, the main TFA was 11*t*-18:1 (averaging 125 mg/100 g meat). On the contrary, on the first experiment the main TFA was the undesirable 10*t*-18:1,that averaged 36.0 mg /100g meat. Meat from lambs from experiment 2, was fatter and thus had larger amounts of SFA but also of the healthy 11*t*-18:1 and 9*c*,11*t*-18:2 than meat from the first experiment. The meat content 11*t*-18:1 averaged 145 mg /100g and of 9*c*,11*t*-18:2 averaged 40.1 mg/g, what are higher than those of the first experiment (21.2 mg/100g for 11*t*-18:1 and 9.3 mg/100g for 9*c*,11*t*-18:2 (Table 6.2).

In the first experiment, in what to concerns to the absolute contents of TFA, 10*t*-18:1, 11*t*-18:1 and 9*c*,11*t*-18:2, the nutritional value of meat fat was negatively affected by lipid supplementation. The absolute content of TFA and of 10*t*-18:1 increased in meat and no improvement on the the healthy FA (11*t*-18:1 and 9*c*,11*t*-18:2) was observed. In the second experiment, the proportions of 11*t*-18:1 and of 9*c*,11*t*-18:2 on meat lipid profile were high and considering the 3.6 g of IMF/ 100g meat, it may be concluded that the contents in 11*t*-18:1 and on 9*c*,11*t*-18:2 were high in all meats (145 mg /100g meat for 11*t*-18:1 and 40.1 mg/g meat for 9*c*,11*t*-18:2).

To a food could be commercially labeled as a source of n-3 PUFA it must contain at least, 300 mg of 18:3 n-3 per 100g and per 100kcal, or 40mg of EPA plus DHA per 100g and per 100kcal (European Commition, 2010). These targets are simply to high for ruminant meat. For instance, on the meat obtained in the first experiment with 8% of oil supplementation, the 18:3 n-3 concentration only reach 50 mg /100g meat whereas the average concentration of EPA+DHA only reach 12mg/100g meat. Thus, oil supplementation of diets was not enough to attain the minimum value regulamented by the European Commission to consider that the meat from lambs of the present study is a source of n-3 PUFA. Moreover, 100g of meat from lambs supplemented 8% oil only provided 1.6 to 6.5% of the DRV for n-3 PUFA for an adult and the average amount of 11.7 mg/100 g of lamb meat for EPA plus DHA only corresponded to 4.7% of the DRV (Table 6.2).

With the present results we confirmed that is possible, via nutritional manipulation of lipid metabolism, to enhance bioactive healthy FA in meat from lambs reared in intensive system. However, that improvement is almost inexpressive when we consider the modest contribution of the consumption of this meat to the DRVs recommended by official health and nutrition organisms for healthy FA. However, it must be accountered that the amount of fat and FA consumed in a lamb meat dose, will depend on the amount of the serving dose, the

type of muscle/ meat joint and the culinary preparation method. The recommended daily dose of meat is actually 100g, which in the case of *Longissimus* muscle, that corresponds to the loin joint, are aproximaly 4 loin chops, The meat joint, including the amount of intermuscular fat which can be consumed is also a factor that increase the dose of FA ingested and our results only concern to loin muscle and without *epymisium*, thus, with any intermuscular fat. Also, loin muscle is one of the leanest muscles. Finally, the culinary process must be considered to the amount of fat and FA ingested in an eating dose *eg*. grilled vs roasted loin chops.

However, despite the modest contribution to the DRV of healthy bioactive PUFA, it is important to have in mind that lamb meat, even from animals of intensive system, with an improved meat lipid profile, can be an important source of n-3 PUFA and CLA to human populations with a high consumption of this type of meat.

CHAPTER 7

Conclusions, implications and future perspectives

7.1. Conclusions, implications and future perspectives

General conclusions

With the results obtained in the present research, we can conclude that dried leaves and soft stems of *C. ladanifer* can potentially be used as alternative forage source in the formulation of complete, isoenergetic and isonitrogenous diets for fattening lambs. Although *C. ladanifer* can be included up to 15% of DM of the lamb's diet without negative impact on animal productivity or meat nutritional value, meat acceptability can be impaired due to negative effects of on meat tenderness and juiciness. Therefore, in diets containing 50% concentrate, the incorporation up to 10% of DM of *C. ladanifer* seems to be the best compromise. At that level of dietary inclusion, *C. ladanifer* allowed good growth performances, had no effects on carcass composition, sensory traits and nutritional value of meat. Moreover, it was enough to improve the meat oxidative stability assessed by colour and lipid oxidation after 7 days of refrigerated storage.

An inclusion level of 20% of *C. ladanifer* on diets, when associated to lipid supplementation, is probably close to the physiologic stress tolerance of rumen ecosystem, being associated to a high variability in individual growth performance traits.

Concerning RBH and meat lipid composition, the results of inclusion of *C. ladanifer* in oil supplemented lamb diets and with 50% of concentrate were not conclusive. In the first experiment it had a negative effect on meat FA composition, which it was mainly due to the exacerbation of ruminal *trans*-10 shift. As a result, it occured a strong accumulation of 10*t*-18:1 in meat lipids and no change on 11*t*-18:1 and 9*c*,11*t*-18:2. However, these observations were not confirmed in the second experiment, where, inversely, *C. ladanifer* reduced 10*t*-18:1 as well as the total of *trans* FA on meat fat.

Blending linseed and soybean oils (2:1) was an effective approach to improve the nutritional value of meat of concentrate-fed lambs by the promotion of the content of n−3 PUFA up to 72.7 mg/100 g of meat and simultaneously maintaining a low fat content.

Dehydrated citrus pulp can fully replace cereals as the energetic source of the diets for growing lambs fed 50% concentrate. It did not affect growth performance, carcass composition and meat quality characteristics.

The replacement of the cereal by DCP in mixed diets with high lipid level, seems to be a promising strategy to enable the concentration of 11*t*-18:1 and of 9*c*,11*t*-18:2, maintaining low risk of occurrence of *trans*-10 shift. It slightly improves meat nutritional value increasing

18:3 n-3 content on meat intramuscular fat. The design of an experiment with different levels of inclusion of DCP in the diets and with higher number of experimental units is important to explore and to confirm these results.

Implications and future perspectives

Further investigation is needed to understand and to clarify the relationships between the secondary compounds of *Cistus ladanifer* and the subjacent mechanisms that may act and interfere with RBH pathways, meat quality and nutritional value of meat fat.

Despite the ability of tannins to modify the metabolism of unsaturated fatty acids in the rumen and to increase the 11*t*-18:1 and 9*c*,11*t*-18:1 in meat and milk, the inclusion of tanniferous plants in the formulation of diets to improve the fatty acid profile of ruminant products, must be well evaluated and caution is needed. As it was demonstrated with *C. ladanifer* in the present study, the effects on meat lipid composition of the dietary inclusion of a tanniferous plant depend not only on the content of tannins present on vegetable tissues, but also the whole diet formulation, namely the forage:concentrate ratio and the lipid composition, content and its FA intake by the animals.

Much more effort must be applied in the identification of relevant nutritional factors that can regulate endogenous lipid metabolism, which is determined by a complex gene network that encode and regulate the activity of key enzymes, in order to incorporate that knowledge on pratical strategies to manipulate lipid deposition on ruminant products.

Replacing cereal grains by alternative agro-industrial by-products may reduce the dependence of livestock production systems on cereals and simultaneously has the ability to improve meat stability and lipid profile of ruminants' meat. Thus, the exploration of alternative feed sources with potential to achieve these objectives is an important nutritional approach in ruminant's production.

Thus, the research on the use of DCP as replacer of cereals in diets should continue to be explored. It will be necessary to confirm if the desirable effects on meat lipid profile observed by us are consistently reproduced in different dietary conditions (cereals proportion and percentage of DCP incorporation), ruminant species (bovines and caprines) and production target (meat *vs*. milk). The application to beef cattle reared intensively is particularly important to be explored considering the importance of the beef market.

It is clear that the *trans*-10 shift is one of the most important constraints to the improvement of ruminant´s meat lipid profile and that the risk of its occurrence must be reduced. For that, the use of non-starch feedstuffs is surely a potential useful solution. However, it is surprising

how little is known about the occurrence of the *trans*-10 shift. In the next future, it is of high importance to understand the bases that determine the individual variability in animal responses to *trans*-10 shift. Does it is an intrinsic characteristic of the animal, linked to a specific genotype, or does it only depend from the rumen microbioma? Does it result from an interaction between animal genetics and rumen microbioma? If resistance to *trans*-10 shift is genetically determined, what are the genes involved? What is its heritability? Concerning to rumen microbioma composition, does it is specific of each animal, depending on animal genotype or does it only depend of the diet?...

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